Biogeochemistry of Selenium in the Oceans of the Southern Hemisphere

by

Bronwyn Davina Wake

Bachelor of Science/Bachelor of Asian Studies
(Australian National University)

Bachelor of Antarctic Studies Honours
(University of Tasmania)

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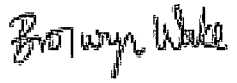

The candidate, Bronwyn Wake, has co-authored the following journal articles, the contents of which are included, in whole or part, in the body of text of this thesis. Bronwyn has been the major contributor to these articles, including carrying out the literature studies, execution of laboratory and field work, interpretation of results, the drafting of the manuscripts and responding to reviewers comments, as outlined by the signed statements held at the School of Chemistry.

Signed: Andrew Bowie, Edward Butler, Paul Haddad (supervisors)

(1) Wake B.D., Butler E.C.V., Featherstone A.M., Virtue, P., Averty, B., Michel, P., 2004. Determination of inorganic selenium species in marine waters by hydride generation - AFS. Australian Journal of Chemistry 57(10): 937-943.

(2) Wake B.D., Bowie A.R., Butler E.C.V., Haddad P.R., 2004. Modern preconcentration methods for the determination of selenium species in environmental water samples. Trends in Analytical Chemistry 23, 491-500.

(3) Wake B.D., Hassler C.S., Bowie A.R., Haddad P.R., Butler E.C.V., 2009. Phytoplankton selenium requirement: the case of temperate and polar regions of the Southern Hemisphere. Journal of Phycology, for submission in January 2010.

(4) Wake B.D., Bowie A.R., Haddad P.R., Butler E.C.V., 2009. The biogeochemistry of selenium along the I9S transect (115 °E) in the Indian sector of the Southern Ocean. Marine Chemistry, for submission in February 2010.

Abstract

Selenium (Se) is a key micronutrient for marine primary productivity in the remote ocean. The element is known to be essential for phytoplankton species growth, but there is limited information on its role and physiological function. This study is the first to investigate basin-scale distributions, speciation and biological requirements of Se for phytoplankton in the various water masses of the Southern Hemisphere, ranging from the subtropical to the polar oceans.

The thesis describes the development of a new shipboard method based on hydride generation with cryogenic trapping and atomic fluorescence detection for the determination of Se species in seawater. A detection limit of 5 pmol l^{-1} Se in a 10-ml sample was achieved, with precision better than 3.5% for Se(IV) standards (0.3–12.7 nmol l^{-1}). Accuracy was determined by recovery studies on natural samples and certified reference seawater.

An ocean transect from Australia to Antarctica along the meridional CLIVAR I9S line (approximately 115°E) was completed in the austral summer 2004/2005. Water column samples were collected in all ocean provinces, with increased sampling in the mixed layer and frontal zones. Results show surface Se(IV) concentrations increasing towards the south. The Se data has been interpreted using ancillary chemical and biological data obtained from the transect.

Laboratory-based cultures were grown to investigate the effect of varying Se concentrations on the productivity and cell health of two temperate and four polar oceanic phytoplankton species. The coccolithophore, *Emiliania huxleyi,* required Se obligately for growth, whereas no effect was observed with the cyanobacterium, *Synechococcus* sp. The effect of Se additions on cell photosynthetic parameters was reflected by an increase in pigment concentrations for three of the polar diatoms. The absolute biological requirement for Se was thus demonstrated in laboratory cultures, with varying requirements among different species.

In order to link the observed oceanic concentrations of Se with the effect on community primary productivity, deck-top incubations were carried out in the oligotrophic waters of the Tasman Sea, which has very low Se(IV) levels in surface waters. Our incubations investigated the influence of additional Se, macronutrients and other trace elements on productivity in this region over a series of four-day experiments. Our full community incubation results differed from those of the single species laboratory cultures, and further work is required to understand the differences before we gain a more detailed understanding of the role of Se in influencing ecosystem health.

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Chapter 1

Introduction

1.1 Introduction

Selenium is an element of environmental, biological and toxicological significance. It is a Group VI element, displaying many properties similar to sulfur, and has properties between those of a metal and a non-metal. It is a metalloid, and like S, Se can display four different oxidation states: -II, 0, +IV and +VI, each with differing behaviour. The two elements have similar ionic radii, redox potentials and electronegativity. However, Se is more polarisable (and therefore more nucleophilic), and there is a strikingly different pH-dependence between thiols and selenols. Selenols have lower pK_a values [1], and are chemically reactive at much lower pH, since Se remains in solution as an ionic form at these lower pHs.

1.1.1 Chemical forms of selenium in the environment

In oxic soils and water, Se is most commonly found in the inorganic forms, Se(IV) as SeO_3^{2-} and Se(VI) as SeO_4^{2-}. However, much of the organic and inorganic complexation chemistry of Se involves the –II oxidation state [2].The stability of Se species is shown in Figure 1.1. Various pe-pH figures can be drawn with differing variables. Figure 1.1 shows species stability for Se activity at a concentration of 0.1 nM, which is most applicable to the marine environment [3].

The pH of soil influences the mobility of Se, with alkaline conditions favouring Se(VI), and more acidic conditions favouring Se(IV) [4]. Se(VI) has similar properties to sulfate (SO_4^{2-}). They are both dominant at high redox potentials, are highly soluble with low adsorption and precipitation characteristics, and directly compete for uptake by plants [5]. In contrast, Se(IV) is dominant at mid-range redox potentials, and its mobility is governed by sorption/desorption processes on solid surfaces, such as metal oxyhydroxides, clays and organic matter [6]. Se(-II) is formed under anoxic conditions which can reduce Se from the inorganic oxic forms to elemental Se (Se(0)), and further to H_2Se and metal- and organically-bound Se(-II) [7]. Se(-II) is stable under strong reducing conditions. Se(0) is formed under various reducing conditions and can be stable over a wide pH range [3] (Figure 1.1). Elemental Se is present as a non-soluble compound, with at least

11 different allotropes [6]. These can display quite distinctive colours (red or pink), and can be seen at the redoxcline in natural waters or sediments [8, 9].

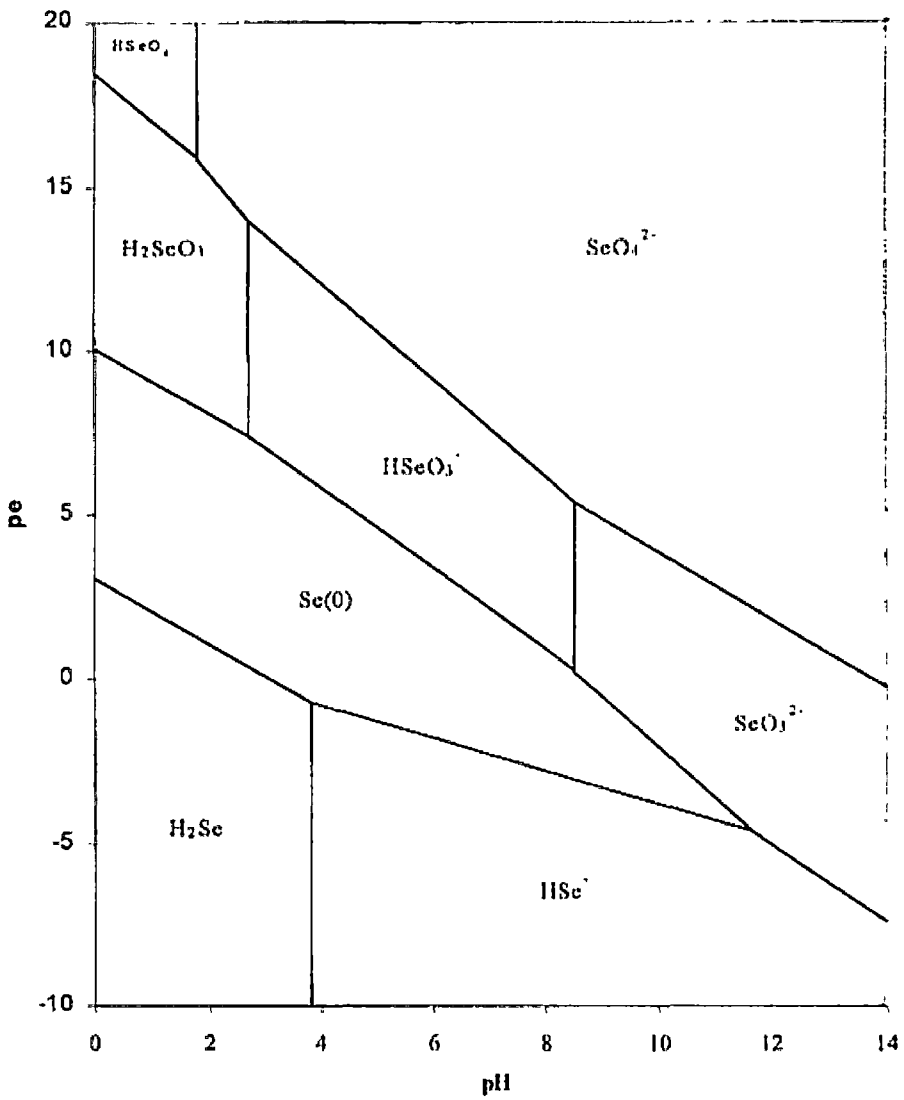

Figure 1.1 Selenium pe-pH diagram at 25°C, 1 bar pressure and zero ionic strength at selenium activity of 10^{-10} mol l^{-1}. Taken from Seby et al. (2001) [3]

In the marine environment, Se is found in: (i) inorganic oxic forms [10]; (ii) organic Se species – a result of biological activity – including Se amino acids (typically analogues of the S amino acids) [11], and volatile species (such as dimethylselenide (DMSe) and other analogues of S species) [12]; and (iii) particulate and colloidal forms of Se(0) [13].

1.1.2 Role of selenium in biological systems – toxicity and essentiality

Selenium is known to be essential for biological systems, but at levels only three to five times above bio-essential concentrations it can also be toxic [14]. It has the narrowest biological tolerance range of all the elements [15]. The behaviour and reactivity of Se depends on its oxidation state and, in general, inorganic forms of Se are more toxic than organic forms. For humans and most other mammals, the toxicity of inorganic Se increases in the order Se(IV)<Se(VI)<hydrogen selenide (H_2Se) [16, 17]. Toxicity occurs when Se substitutes for S in sulfur-containing amino acids (cysteine and methionine). The substitution of Se changes the behaviour of the amino acids (and therefore proteins into which they are incorporated) [18]. When Se replaces S in the cysteine molecule, the resulting protein function is affected, along with the ionisation pH of the selenol group. Reactivity is increased in comparison to the thiol group [1]. Replacing Se for S in methionine has less of an impact, but can influence the enzyme activity if the replacement is near the active site of the molecule [19].

The importance of Se to human health was first recognised in the late 1950s. Maintaining an adequate Se balance is reported to enhance DNA repair processes and improve immunological mechanisms [20], as well as protecting against metal toxicity from other elements, such as mercury [21]. Deficiency can cause heart disease, hypothyroidism, decreased immune response [5] and, in extreme cases, juvenile cardiomyopathy (Keshan Disease) [4]. Se deficiency is also thought to play a role in chondrodystrophy (Kaschin-Beck Disease) which causes enlarged joints, shortened fingers and toes and, in the extreme, dwarfism [4]. It has been estimated that ~500-1000 million people worldwide are Se deficient (2001) [4]. Humans require at least 40 μg Se d^{-1} to maintain maximum expression of Se enzymes (20 μg Se d^{-1} can prevent Keshan Disease), and possibly up to

300 μg Se d^{-1} to decrease cancer risks [4]. The recommended safe upper limit is 400 μg Se d^{-1} (World Health Organisation), although it is recognised that this may be conservative. Eating a single Brazil nut could exceed the recommended daily amount for Se [22]. Excess Se can cause loss of hair and nails. Se accumulates to a higher degree in organs compared to muscle tissue [23].

In humans, many (at least 25) Se-containing enzymes are known, although the function of some remains unknown [19]. Glutathione peroxidase was the first enzyme identified with Se as an essential component. It is an antioxidant that protects cells by reducing hydroperoxides and free radicals. Se-cysteine is commonly regarded as the 21st amino acid because of its role in glutathione peroxidase [1]. The selenol group of Se-cysteine plays a fundamental role in the protection against free radicals, whilst Se-methionine is involved in the protection of cells from radiation and light damage [19].

1.1.3 Sources and cycling of selenium

Selenium is ubiquitous in the environment. It is commonly found as a secondary component of heavy metal sulfides because of the similarity in chemical properties of the two elements [24, 25]. The average abundance of Se in the crust is 0.05-0.09 mg/kg, about 1/6000th that of sulfur and of similar abundance to cadmium and antimony [20]. Its geographical distribution is highly variable, with low Se belts identified along the mid-to-high latitudes in both hemispheres [26]. There are regions with extremely high and extremely low Se soil concentrations. Soils are typically 0.1-2 μg Se kg^{-1} [4], but there are regions that are Se-deficient (<0.1 μg Se kg^{-1}) such as Denmark, Finland, New Zealand, eastern and central Siberia and a belt from northeastern to south central China, and regions with seleniferous soils (>0.5 μg Se kg^{-1}) such as the Great Plains of the United States of America and Canada, Enshi County China, India, Columbia, Venezuela and parts of Ireland. These seleniferous areas can have concentrations up to 1200 mg Se kg^{-1} [20], but the distance between Se-deficient and seleniferous regions can be as little as 20 km, most notably in China and Brazil [19]. Australia is also reported to have a wide range of Se concentrations. Many areas are Se

deficient, with some farm lands (grazing pastures) supplemented with Se fertilisers to ensure the health and quality of stock [27].

There are no significant Se ores or ore deposits. Se is primarily produced as a by-product of copper electrolytic refining [19]. Se can be present in high concentrations in coal, up to 43 mg Se kg^{-1} in high S coals (found in parts of USA, Russia and China), whilst the world average is 1.6±1.0 mg Se kg^{-1} for hard and brown coal [19]. Coal can be Se-enriched up to 65 times that of the surrounding soil, the highest enrichment of all elements [28]. The mining, processing and combustion of coal represent the major mobilisation of Se in the natural environment.

Natural atmospheric sources of Se include crustal weathering and the resulting dust (inorganic and particulate Se), volcanic emissions (inorganic Se), sea-salt spray and emissions from the continental and marine biosphere (organic Se). The marine biosphere is thought to be a dominant source of Se, contributing 60-80% of the natural emissions [29], and helping to balance the global Se budget [30]. Terrestrial biological emissions are dominated by dimethylselenide (DMSe), while marine biota produce a range of methyl selenides – predominantly DMSe and a mixed S and Se compound, dimethylselenidesulfide (DMSeS) [31].

Anthropogenic Se is estimated to contribute 37-40% of total Se to the atmosphere, with coal combustion alone accounting for ~50% of the anthropogenic emissions [29], the majority of which are released in the latitudes between 30°N and 90°N. Wet and dry deposition returns atmospheric Se to both the terrestrial and marine environment. Inorganic selenium is also released into the environment as the result of a range of human activities: fossil fuel combustion, fly ash leaching, agricultural drainage, nonferrous metal smelting, and sulfide ore, coal and phosphate mining [18, 28].

In the atmosphere, Se volatiles are likely to be subjected to similar transformations and processes as S volatiles, although the concentration of DMSe is four orders of magnitude less than DMS [30]. Whilst DMS has been extensively studied due to its role in climate [32], DMSe has received less

interest. In the North Atlantic, there is a positive relationship between DMS and DMSe, indicating both are likely to be by-products of algal processes [31]. The low concentration of Se relative to S means it may have a trivial influence on atmospheric properties [30], although marine volatile Se species may have an influence on the terrestrial environment since the transportation from the ocean to land may be a source of Se to Se-depleted regions, possibly analogous to the cycle of iodine [33], and thus be important for human health.

1.1.4 Selenium in the aquatic environment

In the marine environment, Se is found predominantly as the inorganic oxyanions of Se(IV) and Se(VI); it can also be found as elemental Se (Se(0)), as well as organic Se (Se(-II)). The term 'organic Se' includes Se-amino acids (such as Se-methionine, Se-cysteine), Se-proteins, Se-lipids, methylated volatile Se compounds and the trimethylselenonium ion [34]. The total Se concentration is low (typically less than 1.5 nM), with individual species being less than 1 nM. In freshwater systems, total Se concentrations vary depending on the system. Waters with anthropogenic inputs are often at toxic levels (for fish >60 nM [19]) whilst clean systems are influenced by the geology of the region and can also vary greatly [35].

Organic Se is found typically only in the upper levels of the oceanic water column and is a result of biological activity. Se(IV) species are taken up preferentially by living organisms in the marine environment [13] and are converted into organic Se, which is the biochemically functional form (Figure 1.2).

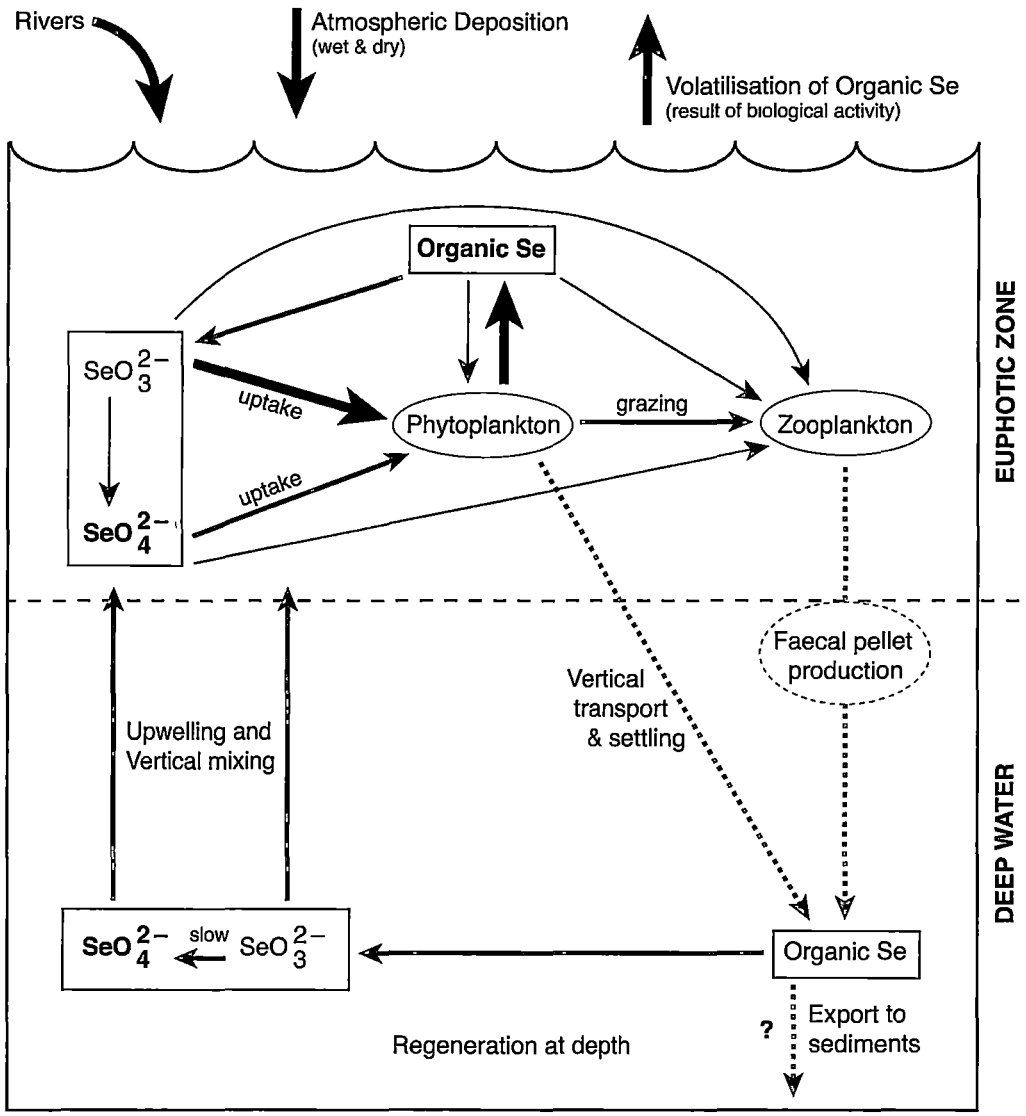

Figure 1.2 Schematic diagram of the biogeochemical cycling of selenium in the marine environment. The dominant species and processes in each region are indicated in bold.

Oceanographic studies of Se distribution have predominantly been in the Northern Hemisphere. The Southern Hemisphere has a lack of measurements, making it difficult to discern much information on Se behaviour in the waters of this region, and whether inter-hemisphere differences exist related to sources and biological processes. The Southern Ocean is a very different environment to the other ocean basins, with minimal anthropogenic inputs and active formation of new water masses. It is also a high-nutrient, low-chlorophyll (HNLC) region [36]. Low chlorophyll indicates low phytoplankton biomass and suggests low primary productivity, which in these regions is not limited by a lack of macronutrients. What limits productivity is not established unequivocally: factors include low iron [37-41]; light limitation; and/or a deep mixed layer [42]; with low silicate possibly limiting diatom production in some areas [43]. While iron has been recognised as a major limiting factor, because of its multi-faceted role in the algal cell[1], the role of many other trace elements remains relatively unknown, although some likely functions have been elucidated [45].

Laboratory studies have shown Se to be an essential micronutrient for some phytoplankton species and it is routinely added to many culture media [46-48], but the role it plays is yet to be defined. Whilst Se is recognised as a micronutrient and has been investigated in the laboratory for interactions with phytoplankton, this has not been followed by studies on how it could affect natural phytoplankton communities. The availability of Se may influence the speciation of phytoplankton found in an oceanic region, as has been demonstrated for the tiny eukaryote, *Ostreococcus* [49]. *Ostreococcus tauri* has a gene for a Se-protein, whilst *Ostreococcus lucimarinus* has the same gene except Se-amino acids are replaced by non-Se amino acids. Since Se is seen to be required by some but not all phytoplankton, certain phytoplankton species in ocean regions with low Se concentrations may suffer limitation.

[1] A large number of Fe atoms (22 or 23 atoms [44]) are required for the photosynthetic system and Fe is also required as an enzyme cofactor, necessary for the intracellular metabolism of nitrogen [45], with no replacement element yet known [46].

1.2 Aims of this thesis

This thesis combines three separate, but linked studies, into Se in the marine environment: (i) the distribution and speciation of Se in the open ocean; (ii) the effect of Se on individual phytoplankton; and (iii) the effect of Se on a natural mixed phytoplankton community.

Chapter 2 outlines the method development, enabling the measurement of the individual Se species at the low concentrations found in the marine environment. It covers the analytical technique employed, methods used to overcome interferences, the conversion of Se(VI) and organic Se to Se(IV) (which is essential for its measurement), and preconcentration methods (which increase the sensitivity of the method).

Chapter 3 details the oceanographic study of Se speciation in the southeast Indian Ocean and Australian sector of the Southern Ocean. The Se data are compared to various oceanographic parameters to discern which of these parameters influence the behaviour of Se.

The role of Se as a limiting micronutrient for different taxa of marine phytoplankton is investigated in Chapter 4. Six phytoplankton strains (two temperate and four polar) were grown in laboratory mono-cultures under varying Se concentrations and measures made of growth, size and photosynthetic activity.

Chapter 5 examines the natural phytoplankton assemblage of the northern Tasman Sea, and how additions of Se (alone and in combination with iron and nitrogen) affect this mixed phytoplankton community.

Chapter 6 summaries the outcomes of the three areas of study and suggests future directions to increase knowledge of the role and biogeochemistry of Se in the marine environment.

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Chapter 2

Analytical method for the determination of selenium species in oceanic waters

2.1 Introduction

This chapter details the analytical method development and is comprised of two sections. The first section details the development of the method for Se species determination at expected natural concentrations, and the second outlines preconcentration methods, with investigation into co-precipitation.

2.2 Method development

2.2.1 Introduction

2.2.1.1 Methods of determination

Selenium is present in environmental water samples as inorganic – Se(IV) and Se(VI) and organic Se (as outlined in Chapter 1). The determination of selenium in natural water samples, including seawater, can be achieved using a variety of methods. Those used for seawater include atomic absorption spectroscopy (AAS) – batch [1] and flow injection analysis [2]; fluorometry [3, 4]; electron capture detection-gas chromatography (ECD-GC) [5]; and differential pulse cathodic stripping voltammetry (DPCSV) [6, 7].

Several analytical methods are suitable for the speciation of Se and these are discussed briefly below. AAS is a well-established technique, with introduction of the sample being possible in a number of ways. Hydride generation is commonly used for Se work [17-19], and it can be used in batch [1], flow-injection [20] and continuous-flow [21] modes. Se hydride is generated by reaction of Se(IV) with borohydride, but Se(VI) will not react to form the hydride. Direct injection of the sample, for total Se species determination, into a graphite atomizer has also been used [22]. DPCSV is an electrochemical method that determines Se(IV) by the deposition of a HgSe film on a hanging mercury drop electrode. Dissolved organic material affects the efficiency of this method [23]. Fluorometry uses the ligand, 2,3-diaminonapthalene or one of its derivatives to form a fluorescent complex with Se(IV). Only Se(IV) will react to form the complex so other Se species need to be transformed to Se(IV) for detection by this method. GC analysis of Se often takes advantage of the same Se complexes as those used for fluorometry. GC of these complexes has been coupled to electron-capture

detection [5]. Alternatively, volatile Se compounds, for example dimethylselenide, can be directly determined by GC. However, concentrations of these species are low in the environment and normally sample preconcentration techniques are required. Other possible methods for Se analysis are inductively coupled plasma-mass spectrometry (ICP-MS) and atomic fluorescence spectrometry (AFS). ICP-MS is capable of determining all Se species without significant sample pretreatment. This method is potentially sensitive and can be used for multi-element detection. However, Se has a high ionization potential, which reduces the sensitivity of the method [24] and the most common Se isotope, ^{80}Se, suffers from isobaric interferences [25]. Use of a dynamic reaction cell or collision cell with ICP-MS improves the sensitivity for Se [26, 27]. AFS is used with hydride generation for Se determination with atomization in a flame. AFS has been reported to be three orders of magnitude more sensitive than AAS, when using the same experimental set up [28]. Of the possible analytical methods discussed above, AFS is well-suited for shipboard use as it is a compact system that does not have special operational requirements [29]. AAS and ICP-MS both have large space requirements, while DPCSV is affected by movement. Fluorometry involves lengthy sample treatment and is prone to analytical errors [13].

AFS offers significant advantages in terms of linearity of dynamic range, detection limits and size when compared to other traditional methods [30]. Early work with Se and AFS was carried out by Thompson (1975) [31]. This work obtained a detection limit of 0.76 nM Se. AFS has not been used as commonly as AAS, because no commercial instrument was available until the early 1990s [32]. Recently, AFS has been used predominantly for analysis of the inorganic Se species but also for the volatile organic Se species, for example dimethylselenide, [33, 34] and Se-amino acids [35]. Some methods couple a chromatographic step, typically HPLC, with flow injection and an on-line reduction step to determine inorganic Se and Se-amino acids simultaneously [36, 37]. Whilst there are reports of batch hydride generation coupled with AAS detection [1, 17], most work with AFS has been coupled to continuous flow and flow-injection hydride generation. Use of continuous flow and flow-injection AFS for seawater analysis has given detection limits at the <0.1 nM level [38, 39]. These levels of detection are in the

range applicable to natural marine waters. However, batch hydride generation offers the advantage of allowing larger sample volumes and preconcentration, by use of a cryogenic trap, to improve detection limits. It also separates the H_2Se from the excess generated hydrogen, making the peak easier to detect.

Most methods are only capable of direct determination of Se(IV), so other Se species need to transformed to Se(IV) before they can be measured.

2.2.1.2 Selenium(VI) determination

Se(VI) is calculated by subtraction after separate determination of total inorganic Se (Se(IV) + (VI)) and Se(IV). Most analytical techniques, including hydride generation, are capable of determining only Se(IV) directly, since only Se(IV) can form SeH_2 by reaction with $NaBH_4$ (except under very acidic conditions) [43, 44]. To measure total inorganic Se an appropriate reduction step is needed. There are many potential reducing agents but few are suitable for the reduction of Se(VI) to Se(IV). Reductants that have a redox potential lower than the redox potential of the Se(IV)/Se(0) couple are not suitable for use as in their presence Se(VI) is reduced to Se(IV) and then Se(0), which is then precipitated. Even mild reductants, such as ascorbic acid and sulfur dioxide, can cause reduction to elemental Se under acidic conditions [45]. Unsuitable reductants include hydrazine and hydrazinium salts [46], hydroxylamine, thiourea, amino acids and iodide [47]. Other reductants, such as nitrite, as well as V(IV) and Pd(II) compounds, are also unsuitable owing to their interference with hydride formation.

The most commonly reported method for Se(VI) reduction is a heated acid reduction method using HCl at concentrations of 4-6 M. The acid concentration, temperature and time of heating will all affect the efficiency of the reduction (Table 2.1). Use of a heated HCl reduction has been investigated by many researchers but consensus on its suitability has not been reached. The acid concentration is most commonly between 4-6 M but up to 9 M HCl [48] has also been used. The heating times vary from 5-6 min [1, 49] to 4 h [50], with 15-30 min commonly chosen [18, 24, 51, 52]. Temperatures also vary widely, ranging

from 60°C [50] to 140°C [21] with 85°C to boiling point being popular [2, 53] (Table 2.1).

Table 2.1 Reported conditions for heated HCl digest for selenium(VI) reduction

Acid concentration	Temperature	Time	Conditions	Reference
5.6 M	90°C	30 min	Open vessels	[54]
4 M	Boil	15 min	Covered with watchglass	[55]
6 M	60°C	4 h	Conical flask	[50]
5 M	85°C water bath	Varied *	Sample analysed immediately	[2]
6 M	70°C	6 min	Sample analysed immediately	[49]
6.8 M	Boiling water bath	48 s	On-line system	[56]
9 M	150°C	10 min	Closed vessel	[48]
4 M	Boil	10 min	NR	[53]
6 M	100°C	20 min	NR	[24]
6 M	140°C	NR	On-line system, ice bath cooled	[21]
6 M	100°C	15 min	NR	[52]
4 M	130°C	1 hr	Al block heater	[57]
5 M	95°C	15 min	NR	[18]
5 M	Boiling water bath	30 min	NR	[51]
5 M	Boiling water bath	20 min	Bring back to 25ml after cooling	[58]

NR = not reported

* ~10 min for Se(VI) reduction; 180 min for organic Se (Se(-II)) conversion

An alternative reaction is the use of bromide in an acidic medium. Bromide is a stronger reducing agent and is more effective at reducing Se(VI) to Se(IV) [59], (about 19 times more effective than chloride [47]). The use of bromide for Se(VI) reduction is less commonly used throughout the literature, but there have been

many investigations into its use (Table 2.2). It has been favoured for on-line use, possibly due to the rapid and efficient conversion of Se(VI) [37, 38, 60-62]. There is also a conflicting report suggesting that organic Se compounds are broken down when the sample is heated for extended periods [63]. Brindle and Lugowska [47] report on extensive investigations to determine optimal but mild conditions for Se(VI) reduction. Their outcomes were pursued further in these investigations.

Table 2.2 Reported conditions for selenium(VI) reduction by bromide reaction

Reagent concentration	Temperature	Time	Conditions	Reference
1.8 M HBr	70°C	50-120 min	Se-met reduced	[63]
5% KBr & 50% HCl	200°C	NR	On-line system	[37]
30% w/v NaBr & 3 M HCl	NR	1 min	On-line system, 210 W microwave	[38]
0.42 M NaBr & 1.6 M HCl	70°C	1 h	40 ml sample	[39]
5.8 M HBr	100°C	63 s	On-line system	[62]
25% KBr & 5 M HCl	160°C	NR	On-line system	[61]
10% HBr & 10% HCl	NR	28 s	On-line system, 100 W microwave	[60]
0.18-0.54 M HBr &1.2 M HCl	Boil	15 min	Se(0) will effect value if present	[64]
1.5% KBr & HCl	Boil	1 h	NR	[65]
0.75 M KBr & 1.2 M HCl	NR	NR	20 ml sample	[66]
0.3 M KBr	90-100°C	25 min	400 ml sample, pH 1 – adjusted with HCl	[67]
1.4 M KBr & 0.48 M HCl	Boiling water bath	17 min	10 ml sample	[47]
2% KBr & 4 ml 1HNO_3/3HCl	60°C	30 min	Milk samples, 2 g, dilute to 50 ml	[68]
4% KBr & 1 M HCl	70°C	1 h	NR	[69]
0.84 M KBr & 1.2 M HCl	85-90°C in water bath	25 min	20 ml sample	[70]

NR = not reported

2.2.1.3 Total selenium determination

Total Se can be determined after an oxidation step to convert all Se to inorganic Se, and it may need to by followed by a reduction step to convert all Se present to Se(IV). Organic Se compounds are very resistant to acid attack and as such a very potent reagent (acidic and oxidising) is needed to convert them to Se(IV) [16]. The method chosen must not interfere with hydride generation, so nitric acid and other strong acids are not preferred. Methods for total Se are quite varied, with UV irradiation, heated acid/persulfate and heated bromine reaction being popular.

2.2.1.3a UV irradiation

UV irradiation is favoured by many authors, but again conditions vary greatly in reports. Some methods claim that only Se(VI) is converted to Se(IV) [71, 72], whilst others report that organic Se species are also converted into inorganic Se species [73, 74] but not necessarily Se(IV). Investigating the reports for organic Se conversion, Cabon and Erler [2] state that the conversion of Se species is very dependent on pH, with higher pH yielding greater recoveries [71, 72]. Chemical additions to the sample to improve conversion efficiency also vary. Some authors add hydrogen peroxide to assist the breakdown of organic material [73-75], while Mattsson and colleagues [76] found it was not necessary except for samples purged of oxygen or when DOC exceeded 25 mg C l^{-1}.

2.2.1.3b Persulfate oxidation

Persulfate oxidises all dissolved non-volatile Se species to Se(VI), and by adding a reduction step, total Se can be determined [77]. The reaction has been reported with 1 ml of 2% (w/v) potassium persulfate in 4 M HCl, boiled for 1 h [78]. Using this acid concentration, the Se(VI) reduction simultaneously occurs. Zhang and colleagues [79] showed this reagent to give complete decomposition of organic Se compounds even with 15 mg ml^{-1} organic compounds present. Little work has been done on testing the validity of this method and most work has tended to replicate the early work in this area or to incorporate only minor adjustments.

2.2.1.3c Hydrogen peroxide oxidation

Hydrogen peroxide has also been reported to oxidize organic Se compounds [79]. This has been reported in a heated reaction [80] but more commonly as an

addition during UV irradiation. The use of peroxide, like persulfate, requires a reduction step to ensure all Se is present as Se(IV).

2.2.1.3d Bromine oxidation

Bromine can be used to break down organic Se compounds, since it has a great affinity to cause oxidative addition to these compounds, allowing cleavage of the C-Se bond under relatively mild conditions [81]. This reaction will convert all Se present in the solution to Se(IV) using HBr and bromine (generated in situ from $KBrO_3$) and heated at 150°C [82]. $KBrO_3$ needs to be present to provide sufficient bromine for the complete conversion of organic Se compounds [83]. This method has been shown to efficiently convert organic Se compounds (namely trimethyl selenonium ion [81], Se-methionine, Se-ethionine and Se-cystine[82, 83]) to Se(IV). However, it has the disadvantages that bromine is a possible interferent in hydride formation and the method is susceptible to matrix interferences [84]. No further investigations into this method were carried out for these reasons.

2.2.1.4 Preservation of samples

The determination of Se in environmental samples is rarely carried out in the field, with samples normally being preserved for later analysis. Preservation methods vary, with acidification, refrigeration and dark storage being preferred. The stability of Se speciation in natural samples is largely unknown, although established methods are used for storage (further discussed in Chapter 3), plus there is the potential for loss of analyte from the sample. It is important to consider these issues for stored and acidified samples.

It is difficult to discern the best preservation methods as many of the reported stability and preservation studies use elevated Se concentrations (an order of magnitude or more above natural ocean levels) and artificial matrices [8-11]. Freezing of samples has been reported as one of the best methods to preserve speciation [12] but this presents some problems. Samples need to be filtered before freezing or rupture of cells can result in anomalous results [13, 14]. Freezing also requires large storage space and care to ensure samples remain frozen until analysis. Acidification is widely accepted for species preservation,

however, excessive acid can change the speciation in the sample [1, 9, 10, 13]. Chloride has been seen to stabilise the inorganic species, so HCl is the preferred acid [10]. Storage of samples is often dictated by convenience. While it has been reported that Teflon and borosilicate glass materials are preferable to polyethylene [15], it is acknowledged that polyethylene bottles are more commonly used due to cost and ease of shipping and handling [16]. Ideally, samples will be analysed as soon as possible after collection. On long ocean voyages this requires an instrument suited for shipboard use. The methods used previously for seawater analysis have typically been shore-based. However, some of these methods could also be suitable for shipboard use.

The work outlined in this chapter describes a method for direct determination of selenium in marine waters at environmental levels. It builds on an existing method used at sea for arsenic analysis [40, 41]. The method couples batch hydride generation and cryogenic trapping with AFS detection (HG-CT-AFS). This method was initially adapted for selenium analysis as part of an earlier project [42], however, additional development was necessary, including further adaptation of the technique for Se(VI) and total Se determination, improving the precision at low natural Se levels, and thoroughly evaluating the possible interferences.

2.2.2 Methods

2.2.2.1 Reagents and standards

All reagents used were analytical grade, unless otherwise stated. Ultrapure water, distilled and purified with a Milli-Q (Millipore) system that had a resistivity of >18 MΩ cm, was used throughout. Stock standards (1000 mg l^{-1} of Se for Se(IV) and Se(VI); 500 mg l^{-1} of Se for Se-methionine; ≡ 12.66 mM and 6.33 mM) were prepared in 30 ml and 125 ml HDPE bottles (Nalgene), which had previously been soaked in 10% v/v HCl and rinsed with Milli-Q water. The stock solutions were refrigerated at 4°C. Dilute working solutions were prepared daily as required. A stock solution of Se(IV) was prepared by dissolving 0.1411 g of HiPure (99.999%) SeO_2 (Spex Industries) in 100 ml of 0.8 M HCl. The reagent was oven dried to remove any moisture before the standard was prepared. The

standard Se(VI) solution was made from 0.2390 g anhydrous sodium selenate (Na_2SeO_4; Ultra grade, Sigma) in 100 ml of Milli-Q water. The standard Se-methionine solution was made from 0.0082 g seleno-L-methionine (Sigma) in 6.6 ml of Milli-Q water.

A 2% w/v $NaBH_4$ solution (AF caplets, 98%; Rohm & Hass) was prepared fresh daily in 0.1 M NaOH (BDH Chemicals). Mallinckrodt AR Select HCl was used routinely throughout the experimental work. A 2% w/v sulfanilamide (Sigma, 99% minimum) solution was made by dissolving the 1 g of sulfanilamide in 1 ml of 12 M HCl and then diluting with Milli-Q water. The solution was kept in the dark at all times.

Reduction of Se(VI) to Se(IV) required 1.67 g potassium bromide (Sigma-Aldrich, 99%+) and 0.4 ml of 12 M HCl (Mallinckrodt); equivalent to 1.4 M KBr and 0.48 M HCl for every 10 ml of sample. A 2% w/v potassium persulfate (BDH Analar) solution was prepared from recrystallised $K_2S_2O_8$ dissolved in Milli-Q water. Hydrogen peroxide (Aristar, ~30% v/v) was added directly to the samples to be UV irradiated (60 μl H_2O_2 per ~80 ml sample).

2.2.2.2 Apparatus

A schematic diagram of the hydride generation-atomic fluorescence system is shown in Figure 2.1 and the operating conditions are summarised in Table 2.3. The detector used was a PSA Excalibur atomic fluorescence spectrometer (PS Analytical, Orpington, Kent, UK, model 10.033). This was fitted with a boosted discharge hollow cathode lamp (Photron, Victoria, Australia, P849SF). Measurements were performed at 196.0 nm, the Se resonance line. The spectrometer was equipped with a solar blind photomultiplier tube, placed at 90° to the incident light, as the detector. A hydrogen-argon diffusion flame formed the atom cell. Data were acquired using National Instruments Labview™ Version 6.1 (Austin, Texas, USA, http://www.ni.com/). Peaks were integrated using the software package ORIGIN (Microcal Software Inc, Northampton, MA, USA).

Table 2.3 Operating parameters of the HG-CT-AFS system

AFS detector		PSA Excalibur
Lamp		Photron BDHCL (Se 196 nm)
Lamp current	Primary	25 mA
	Boost	25 mA
PMT potentiometer		5.50 (≡500 V)
Sample volume		10 or 20 ml
Carrier gas flows	H_2	70 ml min^{-1}
	Ar	400 ml min^{-1}
Supplementary gas flow	H_2	20 ml min^{-1}
Dry air supply		2 l min^{-1}
Reductant		2% w/v $NaBH_4$ in 0.1 M NaOH
Reductant flow rate		2 ml min^{-1} (for 1 min)
U-trap packing material		1) 3% OV-101 on Chromosorb W AW-DMCS, 45/60 mesh
		2) silanised glass wool
Heating wire		Nichrome wire with a total resistance of 1) 4.3 Ω; 2) 3.0 Ω
Heating cycle		0.8 s on/0.2 s off
Data logging frequency		Five per s

All connector tubing used in the system was FEP-lined Tygon® (Cole-Parmer Instrument Co., Vernon Hills, Illinois, USA, i.d. 1/8 inch). All glassware used in the system was made of borosilicate glass. The surfaces of the glassware were deactivated by silylation using the following method. First, the glass was soaked in a 5% Extran (Merck Pty Ltd., Kilsyth, Victoria, Australia) solution for 48 h. After thorough rinsing with Milli-Q water, the glassware was placed in a 10% HNO_3 (BDH Chemicals, Kilsyth, Victoria, Australia) bath for 24 h. Again the glassware was thoroughly rinsed before being placed in an oven to dry for 12 h. The internal surfaces of the glassware were coated with Coatasil Glass Treatment solution (APS Chemicals, Seven Hills, NSW, Australia). This was 2% w/w dimethyldichlorosilane in 98% w/w 1-1-dichloro-1-fluroethane solution. Once the surfaces had been coated and allowed to air dry, the glassware was rinsed with

anhydrous methanol (Mallinckrodt Baker Inc, Paris, Kentucky, USA). The glassware was then placed in an oven to dry at 100°C.

Initial investigations into heated reductions were conducted using a water bath, with samples loosely capped. Routinely, an electronic chilling/heating plate, (Torrey Pine Scientific Inc., San Marco, CA, USA, model IC22) with customised aluminium sample blocks was used. The heater block was set at 100°C and the chiller block at 0°C.

2.2.2.3 Experimental

Briefly, this method [85] used batch hydride generation with cryogenic trapping and atomic fluorescence detection (HG-CT-AFS). Detection limits for Se were in the 5 pM range for a 10 ml sample. The system used was modified from a semi-automated method for shipboard analysis of arsenic species developed by Featherstone et al. (2000) [41]. A number of changes were made to optimise Se determination. The system was controlled using a (300 MHz) desktop PC connected to the interface box via the computer's standard RS232 serial port. The custom interface box provided both the digital and analogue channels required to control and monitor the system. The analogue output signal from the AFS instrument was digitised using a 12 bit A/D converter at a 0.5 s rate.

The control software was written using Labview™. The software used five digital channels to control and monitor the automated system components, time the analytical sequence and log the analytical data. A settings panel in the control software also allowed the operator to customise the timing settings and heating rate for individual runs.

Se(IV) was determined directly (using a 20 ml sample), Se(VI) indirectly (using a 10 ml sample and calculated from Se(IV) and Se(VI) total) after a heated acid-bromide reduction; and total Se after UV irradiation and heated acid bromide reduction. The difference in volume used was to allow for greater sensitivity at the lower concentrations seen when Se(IV) was determined. Details of the choice and optimization of the Se(VI) reduction and total Se determination methods are discussed in the following sections.

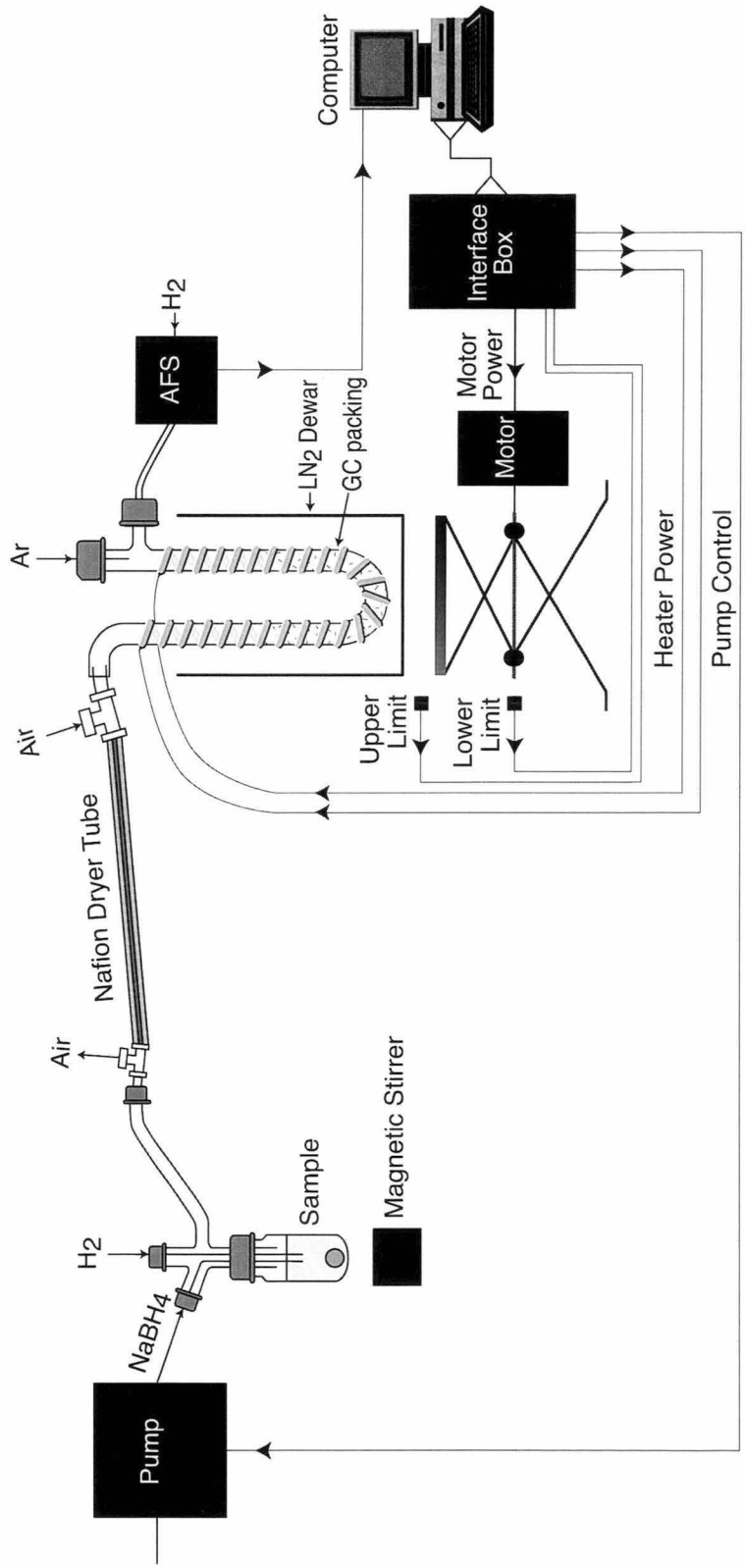

Figure 2.1 Schematic of the HG-CT-AFS system

Prior to analysis each day, the system was conditioned for use. The U-trap was heated at 80% for 10 min with all gases flowing. Two samples of high Se concentration (≡2.53 nM) were run as internal standards, followed by two Milli-Q samples. This ensured the system would provide consistent results from the first run. For analysis, an acidified sample (300 µl HCl for 10 ml sample) was connected to the system. Upon initiation of the computer-controlled sequence, the jack raised a liquid nitrogen dewar flask to cool the cryogenic trap. The motorised jack operated between two cut-off switches. The peristaltic pump was turned on, adding $NaBH_4$ for 1 min to the reaction vessel. At the completion of addition, the pump switched off and a 30 s wait period began, followed by removal of the dewar from the U-trap. Once the dewar was removed the voltage to the heating wire was switched on for 125 s. Data logging began and ended with the computer-controlled sequence. Total time for one analytical cycle was 4.5 min.

For the reduction of Se(VI) to Se(IV), the 10 ml sample with KBr and HCl was placed in a reaction vessel and loosely capped. The reaction vessel was then placed in the heated digestion block (equipped with a electronic chilling/heating plate, Torrey Pines Scientific Inc., San Marcos, CA, USA, model IC22) for 20 min. At the end of this time, the sample was transferred immediately to the chilled block for 5 min and 0.2 ml sulphanilamide was added. The sample was analysed immediately to prevent any back oxidation to Se(VI). Consistent timing was needed to ensure consistent results.

For total Se determination, ~80 ml of sample was placed into quartz glass tubes, which were loosely capped. The samples were irradiated for 6 h by a 1200-W mercury lamp[1]. The samples were left to cool in the UV apparatus. Before analysis for Se, the samples were subjected to the Se(VI) reduction step to ensure all Se was present as Se(IV).

For the removal of nitrite interference, 0.1 ml of sulfanilamide solution was added to an acidified 10 ml sample. It was allowed to react for 4 min before analysis.

[1] Samples were weighed before and after irradiation to monitor loss by evaporation.

For reduced samples, the sulfanilamide was added when the sample was removed from the heat and placed to cool.

2.2.2.4 Modifications for shipboard use

Some minor changes were made to the system to enable shipboard use. Hydrogen was supplied by a hydrogen generator (Domnick Hunter Ltd., Gateshead, UK, model 40H). The heating cycle was adjusted to 0.9 s on/0.1 s off for 130 s, to ensure complete drying of the U-trap packing between runs. Sample volume for Se(IV) determination was altered to 20 ml to increase LOD, the volume of HCl was increased to 0.4 ml per sample and sulfanilamide to 0.2 ml per sample.

2.2.3 Results and discussion

2.2.3.1 Interferences

It has been reported that transition metals, nitrite [86] and other hydride-forming elements, in particular arsenic, can interfere in the determination of inorganic Se by hydride generation. The effects of nitrite and arsenic on the recovery of Se were therefore investigated. Nitrite was found to interfere at concentrations as low as 1 μM, but this interference could be removed by the addition of sulfanilamide [50, 86]. Sulfanilamide (2% w/v, 0.1 ml per 10 ml sample) was effective at concentrations up to 4 μM but did not quantitatively restore the signal at 5 μM nitrite.

Arsenic was tested for interference in the detection of Se. As(V) and As(III) were both tested and shown not to interfere at elevated environmental levels, for example at concentrations up to 0.27 μM As(V) and 0.04 μM As(III). These concentrations were much higher than either species would be seen in clean marine waters. Concentrations have been reported in the Southern Ocean as a maximum of 25 nM for As(III+V), with As(III) levels being less than 0.04 nM [87]; and in the North Pacific, As(III+V) at a maximum of 23 nM and 0.07 nM for As(III) [88].

No further studies were undertaken to determine if trace metals would interfere in the method. Their levels in uncontaminated marine waters are not expected to

interfere [39, 89]. Samples from the Derwent River estuary, Tasmania (an estuary severely degraded by metal discharges from industry over 90 years [90]) were spiked with inorganic Se and comparison of linearity with calibration curves suggested that no other interference was present, even in these samples.

2.2.3.2 Reproducibility and accuracy

The reproducibility of the method had previously been determined as a percentage relative standard deviation (% RSD) from eight measurements using 1.27 nM and 12.7 nM standard solutions. It was found to be 3.2% at 1.27nM, and 2.2% at 12.7 nM. Further work was done to determine the reproducibility at lower levels, and at 0.32 nM (n=8) it was found to be 2.7% RSD.

The accuracy of the method was evaluated by analysis of a certified reference material, namely open-ocean water NASS-5 (National Research Council of Canada, Ottawa, Canada). This reference material is not certified for Se but does offer an information value of 0.228 nM [2] (determined by an HG method). Values reported in the literature are 0.167±0.037 nM (n=3) [26] and 0.224±0.024 nM (n=9) [91]. The Se(IV) concentration of the reference sample was determined to be 0.213±0.006 nM (n=5, error is 2s).

2.2.3.3 Selenium(VI) reduction

2.2.3.3a Heated acid reduction

Reduction of the sample in 6 M HCl at 90°C for 15 min was successful for aqueous standards, spiked artificial seawater and spiked deep seawater. However, this method of reduction was unsuccessful when Derwent River estuary samples were used. Reduction with HCl did not result in quantitative recovery of Se(VI) additions. This may be due to organic materials in the sample, which affect the reduction step and cause interference [57, 92].

[2] Only an information value was found for Se in NASS-5, however, Lam and Sturgeon [91], from the Institute for National Measurement Standards, National Research Council of Canada – where NASS-5 is supplied from, report a certified value of 0.228±0.038 nM as well as their measured value. No information is given as to where this certified value came from and no other reference could be found for it.

2.2.3.3b Heated acid bromide reduction

Use of bromide (with 1.4 M KBr/0.48 M HCl) resulted in the quantitative recovery of Se(VI) in spiked Derwent River estuary samples (Table 2.4). As the bromide reduction uses less acid and is a milder reaction, it was less likely to cause loss of analyte or destruction of the silation coating of the glassware. It was also unlikely to affect any organic Se species present in the sample and this is discussed later in this chapter (Section 2.2.3.5b).

2.2.3.3c Re-oxidation

There are numerous reports of the spontaneous re-oxidation of Se(IV) to Se(VI) when samples are left at room temperature [54, 56, 80, 93]. It is thought that this re-oxidation may be prevented by purging the sample to removal residual chlorine [80]. To remove the need for this extra step, the samples were analysed immediately after reduction. After heating, samples were placed in ice or the chilled block for 5 min, at which time they were cool enough to be analysed. If samples were not analysed immediately, the results were not reproducible.

2.2.3.4 Application to environmental water samples

The application of the method to environmental samples was carried out to ensure matrix effects did not interfere with Se(IV) determination or the reduction step. The samples tested were: coastal seawater collected near Maria Island, Tasmania; and estuarine water collected at various locations (salinity 32.8, 31.9, 19.0 and 4.7) in the Derwent River Estuary, Tasmania. Analysis of these samples was carried out for Se(IV) and Se(VI) (Table 2.4). All samples had sulfanilamide added to prevent nitrite interference. To ensure that matrix effects did not interfere, the samples were subsequently spiked with either Se(IV), Se(VI) or both and then subjected to analysis, or reduction followed by analysis. All samples were spiked with 0.63 nM of Se. The results of the recovery experiment are summarised in Table 2.4 and indicate that there were no problems with matrix effects for inorganic Se determination.

The successful application of the method to environmental waters of different salinities and sources shows that it is capable of reliable detection of the two inorganic Se species in natural waters.

Table 2.4 Concentration of inorganic selenium species and recovery of inorganic selenium spikes (0.63 nM) from some typical marine water samples

Species	Coastal seawater	Estuarine water (salinity 31.9)	Estuarine water (salinity 19.0)	Fresh river water (salinity 4.7)
Se(IV)	0.06 nM	0.05 nM	0.10 nM	0.08 nM
Se(VI)	0.27 nM	0.23 nM	0.29 nM	0.15 nM
	Recovery of inorganic Se species (0.63 nM addition)			
Se(IV)	101.8%	99.8%	100.1%	99.0%
Se(VI)	100.4%	98.7%	98.2%	99.3%

2.2.3.5 Organic selenium compounds

The presence of organic Se compounds in samples and the effect that each stage of the analysis has on these were investigated. Exactly which organic Se species are found in natural waters is unknown and choice of standards is limited. For this work Se-methionine, which is commonly used in the literature, was predominantly used. Se-methionine is a Se-amino acid.

2.2.3.5a Selenium(IV) analysis

Chatterjee and colleagues have reported that some organic Se compounds, including Se-methionine, previously thought unreactive with borohydride, are capable of forming borohydride active volatile compounds [94-96]. Se-methionine recovery was reported to be 19.7% at low borohydride concentration (0.3% w/v), with recovery decreasing as the borohydride concentrations increased. To test if this behaviour occurred under the conditions used in the present project, solutions were spiked with Se-methionine and subjected to standard Se(IV) analysis. Chatterjee et al. [97] propose that Se-methionine forms diethyldiselenide from the reaction with borohydride, so this should be seen as a separate peak from the main selenium hydride peak, due to their well-separated

boiling points. No additional peaks were seen or increases in peak height, even when the samples were spiked to a Se high level (1.27 nM and 12.7 nM). Under the conditions used, it appears that Se-methionine will not be oxidised to Se(IV). With the proposed decomposition mechanisms for the organic Se compounds [97], any compounds formed by Se organic compounds would be separate peaks to those formed by the Se(IV) and borohydride reaction. No further investigations were carried out as these peaks would not affect the concentration of Se(IV) determined.

2.2.3.5b Selenium(VI) reduction

The effect of the Se(VI) reduction methods on organic Se species that may be present in natural water samples have been investigated previously [57]. However, HCl or HCl/KBr do not appear to be strong enough reagents to cause significant effect on organic Se species reported. However, many reports were found that use bromine (in particular $HBr/KBrO_3$ or HBr/Br_2) as a method of oxidising and mineralising organic Se compounds (discussed earlier). The amount of bromine in KBr would be expected to be insufficient to cause this conversion. Clearly, a significant proportion of bromine in a higher oxidation number [≥ 0] than bromide [Br(-1)] is necessarily to provide sufficiently oxidising conditions.

Contrary to this, it has been reported that under thermal treatment at 85°C in 5 M HCl medium, organic Se compounds like Se-methionine could be reduced to Se(IV) [2]. To ensure that this was not occurring with the proposed method for the current project, a deep seawater sample was spiked with Se(VI) and Se(VI)/Se-methionine standard solutions and subjected to the chosen HCl/KBr reduction method. No increase was seen for the sample to which the Se-methionine had been added. A natural surface water sample was spiked with Se-methionine and reduced; again no increase in Se concentration was seen compared to an unspiked treated sample (data not shown).

2.2.3.6 Total selenium

Investigations were conducted into the persulfate reaction and UV irradiation for total Se determination.

2.2.3.6a Persulfate reaction

The persulfate method of Cutter [98] was tested as this method has been used on open ocean samples [99-101]. Samples were made to 4 M HCl and had a 2% (w/v) potassium persulfate solution added (0.5 ml per 40 ml sample), which was boiled on a hot plate for 30 min. The samples were capped with a watch glass to minimise vapour loss. The method was tested on standard solutions and on natural surface seawater samples (20 ml) spiked with Se-methionine standard solution (at 0.32 nM). Samples were cooled for 5 min before analysis. This method was found to yield irreproducible peaks under the conditions tested. Further investigations were not carried out into this method.

2.2.3.6b UV irradiation

UV irradiation has been reported to convert Se(VI) to Se(IV), but this is thought to be inhibited by dissolved oxygen [102]. When samples are not degassed, a reduction step is necessary to ensure all Se is present as Se(IV) [73]. Analysis of UV irradiated samples that had not been subjected to a reduction step gave inconsistent results. To ensure all Se was present as Se(IV), the bromide/HCl reduction was conducted before analysis of all UV irradiated samples.

Samples tested were natural surface seawater samples spiked with Se(IV) (at 0.32 nM), Se(VI) (at 0.63 nM) and Se-methionine (at 0.32 nM) plus combinations of these. UV irradiation was conducted at natural pH, irradiated for 6 h by a 1200 W mercury lamp in loosely capped quartz glass tubes (~80 ml). The samples were allowed to cool in the UV apparatus. Samples were weighed before and after to account for any loss by evaporation. Results are given in Figure 2.2, which shows the Se peak to be increased after the additional treatment step. When the sample was analysed without KBr/HCl treatment, the lower recovery is evident that not all Se in the sample was present as Se(IV).

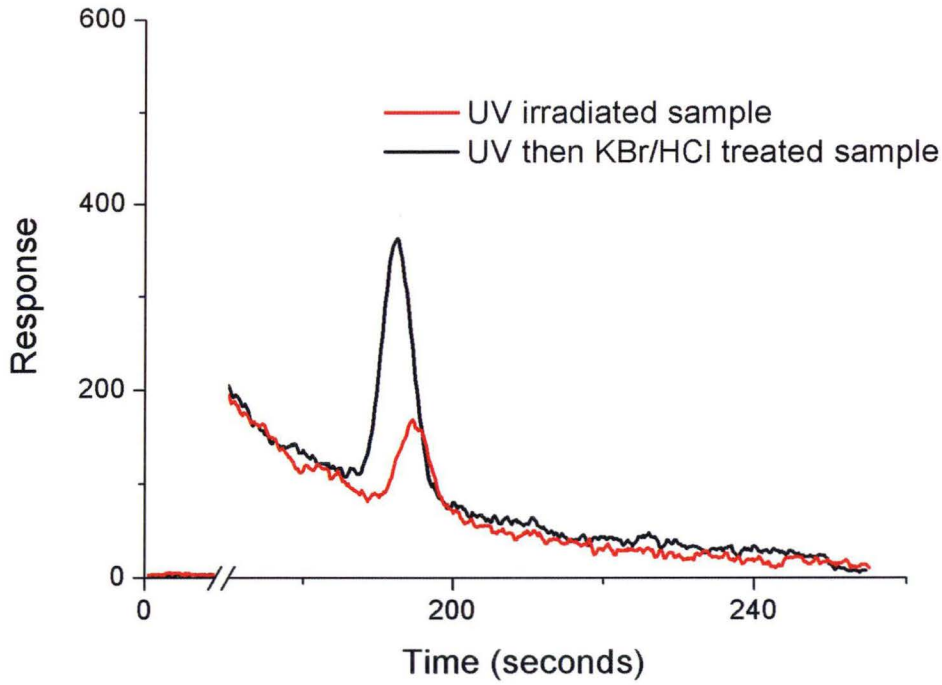

Figure 2.2 Illustration of requirement for KBr/HCl reduction of UV irradiated samples to fully recover selenium from a coastal water sample

UV irradiation of coastal seawater samples yielded Se in excess of that determined from the bromide/HCl reduction. This suggests that under these conditions organic Se species undergo decomposition. The Se standard additions were recovered. Addition of hydrogen peroxide (60 μl) to the sample did not appear to affect the recovery in any way. Peroxide would be needed to help break down organic compounds in suboxic waters, or those with a heavy organic load. In open ocean waters, it is not expected that peroxide would be needed. Addition of persulfate (1 ml of a 2% w/v solution in 100 ml sample) did not change the recovery of Se species after UV irradiation. It was tested on all samples described above (data not shown). No further work was done on this variant of the method.

2.2.4 Conclusions

The HG-CT-AFS method was found to be suitable for Se species determination in marine waters. This robust method was unique in its capacity to operate at sea

while offering low detection limits, good precision and the ability to measure Se(IV), Se(VI) and total dissolved Se concentrations. The methods for determining different Se species were thoroughly tested, as were potential interferences and the reproducibility of the system. The possible need to improve detection limits for Se(IV) are investigated in the next section.

2.3 Sample preconcentration

2.3.1 Literature review

In the marine environment the total Se concentration is low, with the concentration of individual species close to, or below, the limit of detection of most commonly used analytical methods, leading to difficulties in quantifying individual species. Thus, a preconcentration step is often required in order to improve limits of detection. Commonly used methods for preconcentration are: solid-phase extraction (SPE), (including solid-phase microextraction (SPME)); isotachophoresis (ITP); and co-precipitation and cryogenic trapping (CT). Another, more specialised preconcentration approach is electrostacking, which involves on-capillary zone compression and is only used with capillary electrophoresis. This method will not be considered here but its application to Se species has been reviewed recently by Sun et al. [103].

This work presents an evaluation of the above preconcentration methods from their early stages of development to their current status. The benefits of each method and its applicability to the different Se species are considered.

2.3.1.1 Solid-phase Extraction (SPE)

SPE is a procedure in which a sorbent is used to preconcentrate analytes (and usually also to separate them from the sample matrix). SPE can be applied under conditions which preserve the original speciation of the sample and can offer good preconcentration factors leading to lower limits of detection, ease of automation and the possibility of on-line coupling [104]. Most of the previous work for SPE of Se species focuses on the inorganic forms, Se(IV) and Se(VI). In some cases, it is used for preconcentration and removal of matrix effects, whilst

other studies use it for the separation of the two inorganic species through the use of fractional elution. In surface marine waters, the inorganic forms are present as oxyanions, SeO_3^{2-} and SeO_4^{2-} [105], so use of an anion-exchange resin is common for extraction. In contrast, organic species in surface marine waters are primarily thought to be present as Se-amino acids or volatile species and the few reports of SPE of these species have used chelating resins for preconcentration.

SPE methods for the determination of Se are summarised in Table 2.5. Methods used vary greatly depending on the Se species targeted, and limits of detection obtained range from ~30 pM suitable for analysis of unpolluted open ocean waters, to 600+ nM levels. In most cases, the inorganic species (Se(IV) and Se(VI)) are extracted and preconcentrated, although there are also some reports for SPE of the main Se-amino acids (Se-methionine and Se-cystine), total Se and organic Se.

SPE has also been reported for simultaneous preconcentration of both inorganic and organic Se species. Bueno and Potin-Gautier [106] used a chelating and anionic sorbent to successfully preconcentrate Se(IV), Se(VI) and Se-cystine from lake and mineral waters. Separation was achieved by HPLC, with detection by ICP-MS. Se-methionine could only be retained on the resin for standards (albeit at lower recoveries), and not with real samples. This was thought to be due to competition with other ions present in the matrix, in particular hydrogen carbonate ions. Pyrzynska [107] reported another example of coupling different columns to determine inorganic and organic Se species. Chelex-100 chelating resin in the copper form was used for Se-methionine and Cellex-T, a strong anion exchange resin, was used for the inorganic species.

Table 2.5 Solid-phase extraction procedures for preconcentration of selenium species

Species	LOD[a]	Sample	Sorbent	Conditions	Elution	Detection	Ref
Se(IV) Other species converted to Se(IV)	0.127 nM	Seawater	Sulfonated bismuthiol-II loaded Amberlite IRA-400, Cl^- form, 60-80 mesh	adjusted to 2 M HCl 1000 ml sample 8-10 ml min^{-1}	20 ml 0.05 M penicillamine 0.2 ml min^{-1}	HG-AAS	[108]
Se(IV) as piazselenol	~0.03 nM range	Lake and stream water	PD-102-PE, 10 μm diameter	5 ml min^{-1} 5-100 ml	1 ml toluene (2 ml min^{-1})	GC-ECD	[109]
Se(IV)	~0.28 nM range	River water	Fe(III)-Chelex 100 100-200 mesh	pH 4.5 (acetic)	3 ml 1 M NaOH	DPCSV	[110]
Se(IV)	1.01 nM 37.99 nM	Seawater, waste water	dithiocarbamate loaded polyurethane foam	pH 4.5 150 ml sample 2 ml min^{-1}	1 ml isobutyl methyl ketone	ET-AAS ICP-AES	[111]
Se(IV)	0.051 nM	River, lake and seawater	83 mg of 20 μm PTFE fibres	12.4 ml sample on-line formation of Se(IV)-PDC in 1% v/v HCl	1.5 ml 3% w/v KBH_4 in 0.5% w/v KOH	FI-HG-AFS	[112]
Se(IV) Se(VI)	0.127-0.253 nM	None listed	Sulfonated bismuthiol-II loaded anion exchange, 100-200 mesh	0.3-0.5 M HCl 2 M HCl + 0.1 M thiourea	0.02 M cysteine or penicillamine	Spectrofluori-metry	[113]
Se(IV) Se(VI)	~0.1 nM range	None listed	Poly(dithiocarbamate) resin, 200 mg	100 ml sample pH Se(IV) 1.5 Se(VI) 4.8	$3HNO_3$-$2H_2O_2$ v/v	HG-ICP-AES	[114]
Se(IV) Se(VI)	0.063 nM	Tap, lake, surface run-off, well water	Dowex 1×8 Cl^- form, 100-200 mesh	pH 9 100 ml sample	10 ml 0.025 M HCl 7.5 ml 5 M HCl	HG-AAS	[115]
Se(IV) Se(VI)	0.760 nM	Tap war and haemodialysis sample	on-line Dowex 1×8, Cl^- form, 200-400 mesh	600 μl sample 0.5 ml min^{-1}	600 μl 2M CHOOH 350 μl 6 M HCl	FI-HG-AFS	[116]
Se(IV) Se(VI)	2.03-5.32 nM 1.01-2.41 nM	Salt, tap, river and well water	on-line PRP-X100, 10 μm	0.8 ml min^{-1}	80 mM $(NH_4)_2CO_3$	ICP-MS	[117]

Species	LOD[a]	Sample	Sorbent	Conditions	Elution	Detection	Ref
Se(IV) Se(VI)	0.063 nM	Mineral, spring water	Amberlite IRA-400 Cl^- form, 20-50 mesh	2 ml min^{-1}	pH 1	DPCSV	[118]
Se(IV) Se(VI)	0.633 nM	River, rain water (Tested for seawater but too much interference)	Amberlite CG 400 type 2 OH^- form	pH 2.5 (acetic & HCl)	5 ml 0.1 M HCOOH (containing 0.1 ml 2000 mg l^{-1} Pd solution)	GF-AAS	[22]
Se(IV) Se(VI)	10.13 nM 620.57 nM	Tap and ground water	Activated alumina 50-200 μm particle size	natural pH 100 ml sample 4 ml min^{-1}	1 ml 0.1 M NH_3 (2 ml min^{-1}) 6 ml 4 M NH_3 (2 ml min^{-1})	GF-AAS	[119]
Se(IV) Se(VI)	0.060 nM 0.080 nM	River, tap, seawater and drainage sediment CRM	TiO_2	pH 3, 100 ml sample pH 0, 100 ml sample	2 ml of (6 M HCl + 2 mg ml^{-1} $Ni(NO_3)_2$)	GF-AAS	[120]
Se(IV) Se(VI) Total Se	0.152 nM	River, well, rain and spring water	Activated carbon	Reduced to elemental Se by 1 g L-ascorbic acid	2 ml 0.0625 M BrO_3	Spectrophoto-metry	[46]
Se(IV) Se(VI) SeCyst	0.127 nM	Mineral and freshwater (lake)	Amberlite IRA-743	natural pH 100 ml sample 1-1.8 ml min^{-1}	9 ml 1 M $HClO_4$ followed by 18 ml MQ (0.4 ml min^{-1})	HPLC-ICP-MS	[106]
Se(IV) Se(VI) Org Se	0.020 nM 0.018 nM 0.008-11.44 nM	CRM (CASS), sea, lake, river and tap water	SAX cartridge in series with C_{18} cartridge	pH 7-8 1000 ml sample 8 ml min^{-1}	25 ml 1 M HCOOH 25 ml 3 M HCl (5 ml min^{-1}) 2 ml CS_2 (1 ml min^{-1})	GC-MS	[121]
Se(IV) Se(VI) Hydrophobic org Se	0.022 nM (for total Se)	River and lake water	Dowex AG2-X8, 200-400 mesh, Cl^- form Amberlite XAD-8, 20-60 mesh	200 ml sample 4 ml min^{-1}	15 ml 1 M HCOOH 15 ml 3 M HCl	Fluorimetry	[122]
SeMet	0.405 nM	None listed	Cu-Chelex 100 iminodiacetic acid exchange groups, 100-200 mesh	pH 9 100 ml sample 2 ml min^{-1}	8 ml 1.5 M NH_3	ET-AAS	[107]

[a] Limits of detection converted to nanomole per litre from values quoted in the original manuscript

Most of the methods reported have been validated for real matrix applications in fresh or mineral waters [22, 46, 109], with few methods being applied to saline waters. The high ionic strength of seawater causes interference in some SPE methods (e.g. Kubota & Okutani [22]). Sahin et al. [123] used mercapto-silica as the sorbent which was suitable for use with seawater, but only at high Se levels. Dithiocarbamate-loaded polyurethane foam has been shown to be suitable for use with samples up to 3% NaCl [111], although this sorbent was still unsuitable for open-ocean seawater samples (typically 3.5% NaCl).

Gomez-Ariza et al. [121] coupled two columns, an anion-exchange resin and a C18 cartridge, to determine inorganic and volatile Se species. This work was tested with sea, river and tap water, as well as a near-shore seawater certified reference material (CRM). A limit of detection of <0.02 nM for inorganic Se species made it suitable for ambient marine concentrations, when large volumes of sample were used, but under these conditions other compounds and ions in the matrix could overload the column. The determination of volatile Se species is better carried out using a different method because of the poorer reported limits of detection. Cryogenic trapping of these latter species offers much better limits of detection. Amouroux et al. [33] report a limit of detection of 10 fM for dimethylselenide (DMSe) and dimethyldiselenide (DMDSe) using cryogenic trapping with gas chromatography coupled with ICP-MS detection (GC-ICP-MS), compared to 7.6 pM for DMDSe and 5.07 nM for DMSe using SPE with GC-MS detection[121].

As well as the typical SPE phases, there have been other sorbents used. Nanometre-sized TiO_2 particles have been used for preconcentration and separation of inorganic Se species[120]. Se(IV) was adsorbed selectively at pH 3 and Se(VI) at pH 0, giving detection limits below 0.09 nM for both species. This approach was therefore suitable for use with seawater as there was no interference from the major ions present in the sample matrix (Na^+, Cl^-, K^+, Ca^+).

The use of a polytetrafluoroethylene (PTFE) fibre-packed microcolumn, in a flow injection-hydride generation-atomic fluorescence system (FI-HG-AFS), was successful for the preconcentration of Se(IV) [112]. The on-line formation of the

Se(IV)-pyrrolidinedithiocarbamate (PDC) complex allowed the Se(IV) to be sorbed onto the surface of the PTFE fibres. The Se(IV) complex was eluted by KBH_4, which was an efficient eluent, as well as being necessary for the hydride generation step that followed. The limit of detection was 0.05 nM. River, lake and seawater were tested and recoveries of spiked samples were in the range 96-108%. While this work is suitable for use with various water types and has a sufficient limit of detection, it only allows the preconcentration and detection of Se(IV).

SPE resins have not only been used for preconcentration and extraction but also for removal of matrix effects and for storage of the analyte. High levels of dissolved organic carbon can interfere in the chromatographic separation and subsequent detection of Se. This interference can be removed by passage of the sample through a XAD-8 resin prior to the preconcentration step [115]. XAD-8 and XAD-7 resins (acrylic ester polymers) are suitable for adsorption of fulvic acids and will remove organic material, but will not retain inorganic Se[109]. Adsorbents can also be used to store a sample and to preserve the speciation present in the original sample. Here, the sample is passed through the resin immediately after sampling in the field and the loaded adsorbent is then stored for later analysis. Sugimura et al. [124] used XAD resin to adsorb Se(IV) as the Se(IV)-diethyldithiocarbamate (DEDTC) complex whilst at sea. The resin was stored and analysed on return to the land-based laboratory. The acidified water sample which had been passed through the resin was also retained to allow for Se(VI) determination.

An interesting use of a SPE resin was the method by Carrero and Tyson [125] in which Se(IV) and tetraborohydride were retained simultaneously on an Amberlite IRA-400 (strong anion-exchange) column. H_2Se was then generated by the passage of acid through the column, followed by detection using atomic absorption spectrometry (AAS).

2.3.1.2 Solid-phase Microextraction (SPME)

SPME is a relatively new form of SPE [126] in which a silica fibre coated with a polymeric substance of choice is placed into the sample or the headspace above the sample to adsorb the analytes. The fibre is then transferred into a heated injector port, typically that of a gas chromatograph, where the analyte is desorbed, separated and detected. SPME can be used for aqueous or gaseous matrices, with headspace sampling being preferred with low boiling point analytes since some separation from the sample matrix components has already occurred [127]. SPME also is applicable for small sample volumes as the entire sampling device can be miniaturised. SPME is an equilibrium-based extraction method; it does not extract all the analyte from the sample but allows for a distribution equilibrium to be established between the sample and the fibre [128]. The fraction of analyte extracted increases as the ratio of the coated surface volume to sample volume increases.

SPME has been used for determination of a wide range of Se species, normally with coupling to gas chromatography–mass spectrometry (GC-MS) or GC-ICP-MS. In reports to date, it normally produces insufficient enrichment to be advantageous for determination of natural levels of Se in surface waters. One exception has been the method of Guidotti et al. [129] using GC-MS for detection of piazselenol complexes after enrichment by SPME. Here, Se(IV) and total Se concentrations were quantified. Se(IV) was treated to form the piazselenol complex directly, and then the SPME fibre was immersed in the sample for 35 min. For total Se, the sample needed to be treated to convert all Se to Se(IV), which then formed the piazselenol complex. The limit of detection was 0.76 nM (S/N=3).

A comparison of headspace and direct SPME was conducted by Guidotti [130] by first converting Se(IV) to a volatile form (diethylselenide (DESe)) and then extracting the DESe onto a polydimethylsiloxane (PDMS) coated fibre, with detection by GC-MS. Headspace SPME had a reduced sampling time when compared to direct SPME, since diffusion of the analyte was much faster in the vapour phase. For both methods, the solution was buffered to pH 4, since this

provided optimum recovery in the shortest time. Limits of detection (S/N=3) were 1.03 nM (for headspace SPME) and 2.10 nM (for direct SPME).

Vonderheide et al. [131] used SPME for the determination of Se-amino acids (Se-methionine, Se-ethionine and Se-cystine). The Se-amino acids were treated with isobutylchloroformate to increase volatility. A sol-gel coated PDMS fibre was exposed to the sample for 5 min and detection was carried out by ICP-MS. Limits of detection were 0.203 nM for Se-methionine, 0.177 nM Se-ethionine, and 0.367 nM Se-cystine. However, the reproducibility was quite poor (17–23% relative standard deviation (RSD)) and this was attributed to irregularities present in the sol-gel coating of the fibre.

Volatile Se species, such as DMSe and DMDSe, have been determined by SPME coupled with ICP-MS [127]. These species can be separated at ambient temperature in the transfer line by gas chromatographic mechanisms, with no need for external heating. However, temperature needs to be applied for desorption from the fibre and 200°C was found to be the optimum. The SPME fibre (Carboxen™/PDMS coated) was placed in a headspace vial with 5 ml of sample and a stirrer bar, such that there was no contact between the fibre and the sample solution. After equilibrium was reached (25 min), the fibre was transferred to the desorption unit for measurement. Detection with ICP-MS provided greatly improved limits of detection (DMSe 8.87 nM, DMDSe 11.39 nM) compared to detection with AAS (DMSe 0.291 μM, DMDSe 0.393 μM). These limits of detection were a factor of 10^6 times higher than those obtained by cryogenic trapping preconcentration after purging. Cryogenic trapping uses samples of 1 L compared to 5 ml required here. However, the absolute limits of detection are 3.5 ng DMSe and 4.5 ng DMDSe for SPME compared to 0.8 pg DMSe and DMDSe for cryogenic trapping [33].

Coupling of SPME with ICP-MS was used by Mester et al. [132] to determine Se(IV), as well as arsenic, antimony and tin, after hydride generation. H_2Se was collected by headspace SPME extraction on a PDMS/Carboxen fibre at room temperature. By collecting the hydride on a fibre, SPME permitted removal of the excess hydrogen occurring typically with hydride generation. It also offered the

advantage of complete gas-liquid separation, so no moisture entered the detector. However, the limit of detection of this method was only 67.1 nM, thus it did not possess the required sensitivity for use with most environmental samples.

2.3.1.3 Isotachophoresis (ITP)

Isotachophoresis is an electromigration separation method which offers both good preconcentration capability and a high degree of separation of analytes. ITP uses a leading and terminating electrolyte to concentrate a target analyte by forcing the analyte to migrate as a compressed band under an applied electric field. Use of an appropriate pH is most important since this controls the degree of dissociation of the analyte, and hence, its effective mobility. If a suitable pH range is selected, it is possible to separate anions of weak acids according to their pK_a values [133].

The use of ITP for Se preconcentration and determination has not been widespread. Yoshida and Hida [134] reported an ITP method for inorganic Se species using a high pH leading electrolyte containing either a Ni(II)-1,10-phenanthroline complex or Co(III)-ethylenediamine complex. Separation of Se(IV) and Se(VI) was achieved through ion-pairing equilibria between the anions and the metal complex in the leading electrolyte. ITP has also been used for both inorganic and organic Se species. Grass et al. [135] used ITP to preconcentrate Se-methionine, Se-ethionine and Se-cystine using a high-pH electrolyte (in view of the high pK_a values of Se-amino acids). Of the species investigated, Se-cystine gave the best detection limit (1.52 μM), with the detection limit for the two other species being approx. 2.91 μM. Separation was accomplished by coupling the ITP chip with capillary zone electrophoresis using conductivity detection.

Carbonate resulting from the adsorption of CO_2 from the air by solutions with a high pH can cause a problem in ITP preconcentration [136]. However, this can be overcome by choosing a leading electrolyte with a mobility similar to that of carbonate [135]. Inorganic Se species may also be preconcentrated and separated by miniaturised ITP using a planar polymer chip [137]. In this approach, a lower pH leading electrolyte (pH 4.1) can be used, thus avoiding carbonate interference.

The separation achieved was faster than for other reports, allowing a throughput of 8 samples per h. The miniaturised system was limited by physical constraints, only allowing 5.1 μl injection of sample, resulting in a limit of detection of 6.59 μM Se(IV) and 8.23 μM Se(VI). Therefore, it can be seen that, at present, ITP methods for Se generally lack sufficient sensitivity for application to environmental water samples

2.3.1.4 Co-precipitation methods

Preconcentration using co-precipitation methods is used when the concentration of the analyte in the sample is insufficient to permit direct precipitation. The most popular method of co-precipitation functions by the formation of an insoluble metal hydroxide which acts as a collector, forming a colloid with the analyte, and thereby, co-precipitation. Most commonly, lanthanum(III) is used, which precipitates at around pH 9.5 as $La(OH)_3$ and co-precipitates Se(IV) and As(III) [138].

Originally, co-precipitation could only be used with batch methods, as the use of filters to collect the precipitate would increase the pressure in continuous systems. However, the recent use of knotted reactors has allowed collection of the precipitate in continuous flow and flow-injection systems without an increase in flow resistance. The sample solution is mixed with the chosen precipitating reagent and the pH of the sample is then adjusted (usually with ammonia since it is easy to purify and has a high acid buffering capacity) to permit precipitation. After precipitation, the precipitate is collected, dissolved and subjected to final analysis.

Reports of co-precipitation methods apply only to inorganic Se species. Most methods report that only Se(IV) is precipitated, so co-precipitation can be used to remove Se(IV) from the sample and then Se(VI) can be determined subsequently after a reduction step. Tao and Hansen [139] obtained a detection limit of 0.0127 nM Se(IV) from a 6.7 ml sample of tap or well water using lanthanum nitrate (20 mg l^{-1}), followed by quantification using flow-injection analysis coupled to hydride generation AAS (FI-HG-AAS). The precipitate was collected

in a knotted reactor and this work represents one of the first uses of this device with inorganic precipitates. Sun and Yang [140] simultaneously preconcentrated Se(IV), As(III) and Sb(III) by co-precipitation with $Pb(PDC)_2$ (where PDC=pyrrolidine dithiocarbamate) over the pH range 2-4. The PDC reagent was added to the sample, which was stirred for 5 min and the precipitate then collected on a 0.45 μm membrane filter. Quantification was carried out by neutron activation analysis. Se(IV) was determined by this method, giving close to 100% recovery up to pH 5, but Se(VI) did not co-precipitate under any of the conditions evaluated. This method offered good detection limits (0.0127 nM Se(IV)), but required a sample volume of 1 L for real samples. Sea, ground and tap waters were analysed successfully using this approach.

2.3.1.5 Cryogenic trapping (CT)

Cryogenic trapping involves the trapping of volatile species at low temperature, with subsequent release for separation and detection. CT has been used in two ways for Se analyses: (1) trapping of naturally occurring volatile species collected directly onto the cryogenic trap though bubbling of the sample, and (2) trapping of generated H_2Se. CT is applicable only for the preconcentration of volatile species, whether these are naturally occurring or generated.

Cryogenic trapping usually involves a U-shaped tube which is immersed into liquid nitrogen (–196°C), although other liquefied gases have been used [53, 89]. The U-tube is often packed with a substrate to increase the surface area and to thereby increase adsorption [141]. The substrates used for this purpose are numerous, but the most common are various forms of Chromosorb phases (originally used in wide-bore gas chromatography), quartz or glass wool.

The collection of volatile species is achieved using a carrier gas stream. For water samples, an inert gas (typically helium [142]) is bubbled through the sample and then passes through the trap. When used with hydride generation, CT offers the advantage of separation from excess generated hydrogen [143] and preconcentration of the H_2Se, which enables it to enter the detector as a single, sharp peak rather than being spread over the time period for which it is generated

(Figure 2.3). CT typically provides preconcentration factors of approximately 50 [17].

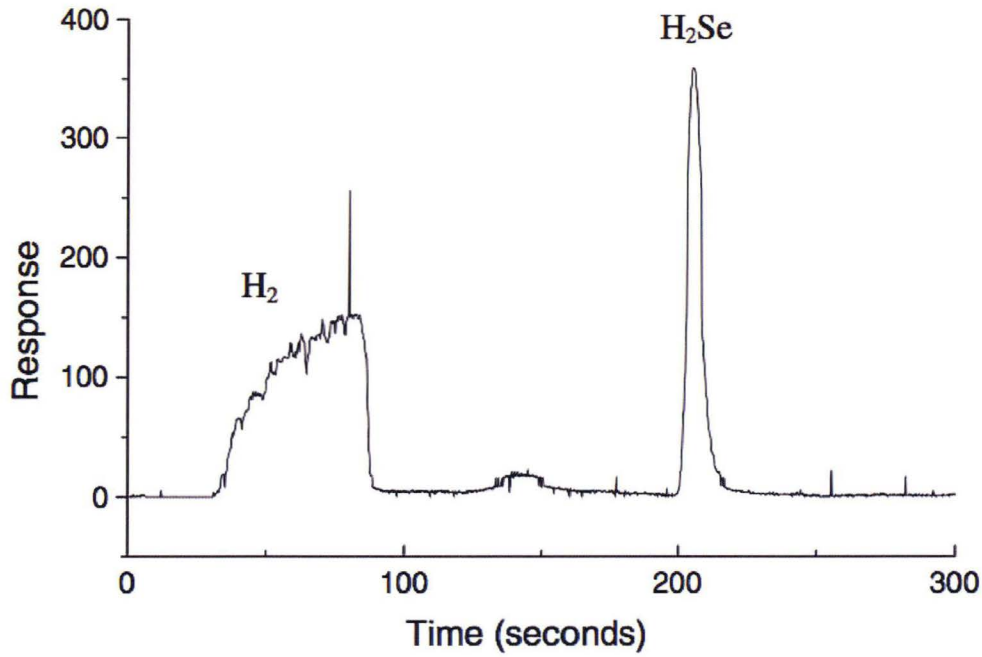

Figure 2.3 Illustration of the cryogenic separation of hydrogen and hydrogen selenide after hydride generation with AFS detection

CT can also be used to separate different species of Se according to their boiling points. As the trap is removed from the liquid nitrogen and warmed, if the species have boiling points that are well separated, they will be released sequentially from the packing to enter the detector. This condition occurs for some organoselenium compounds which have widely separated boiling points, for example, 55°C for DMSe and 155°C for DMDSe.

2.3.1.6 Summary

The low concentration of Se in the aquatic environment makes it difficult to determine this element using traditional detection methods. Use of a preconcentration step allows improved limits of detection, but at the same time

requires larger volumes of sample and more handling steps. Of the preconcentration processes reviewed here, cryogenic trapping allows good preconcentration factors with minimal handling, but it is only applicable to volatile Se species. It also requires a large volume of samples in order to detect the naturally occurring volatile species, but it is currently the best method for their determination. Use of cryogenic trapping for other Se species is limited to those that can be converted to volatile species. Solid-phase extraction has been commonly used and is suitable for the preconcentration of inorganic and some organic Se species. Future research needs to be done to identify and quantify individual organic Se species in water samples, rather than treating these analytes as a single group. Solid-phase microextraction appears to offer significant preconcentration benefits with little sample manipulation. However, the method is also limited to volatile species or those that will absorb onto the limited range of fibres available currently. At the present time, results vary greatly between different SPME methods and detection needs to be carried out with ICP-MS to obtain sufficient sensitivity. Co-precipitation is suitable for the preconcentration of inorganic Se species. Finally, isotachophoresis has not been widely investigated for the determination of Se species and currently does not offer the limits of detection required for environmental water samples.

Co-precipitation was chosen as the method most suitable for investigation. It allows the preconcentration of inorganic Se, in particular Se(IV), which is the biologically important species, and is expected to be depleted in the ocean due to biological activity, possibly bringing it near or below the detection limit of the HG-CT-AFS method.

2.3.2 Methods

2.3.2.1 Reagents and standards

Lanthanum nitrate hexahydrate (Aldrich, 99.99%) solutions were prepared, for 2.5%, 5%, 7.5% and 10% w/v, dissolving the appropriate mass in 20 ml of Milli-Q water. Ammonium hydroxide (28-30%; Sigma-Aldrich) was used to increase the pH of the solutions and cause precipitation. Concentrated HCl (Mallinckrodt AR Select) was used routinely throughout. Four litres of 0.7 M NaCl solution was

prepared (40.95 g NaCl (99.9%, Pronalys Analytical Reagent, Biolab) in 1 L of Milli-Q). An aged deep seawater sample (>1500 m) was used for testing the method. Standards for Se addition are those described previously (refer to 2.1.2.1).

2.3.2.2 Experimental

Samples had 2 ml 5% w/v $La(NO_3)_3.6H_2O$ solution added to 200 ml of sample in a 250 ml HDPE bottle (10% v/v HCl-washed, Milli-Q rinsed). Ammonium hydroxide (~2.5 ml) was added to increase the pH and cause precipitation of $La(OH)_3$. Samples were shaken to ensure complete mixing of reagents and checked to ensure pH~9-10. After 15 min, to ensure complete precipitation had occurred, samples were vacuum filtered (0.45 μm cellulose acetate filters; 10% v/v HCl-washed, Milli-Q rinsed). The filter was rinsed with Milli-Q (three rinses). The filtrate was retained for analysis. No precipitate was seen to have passed through the filter.

To recover the Se(IV), the filter was placed in a reaction vessel, where 300 μl HCl and 5 ml Milli-Q was added to dissolve the precipitate. The filter was then removed with plastic tweezers (10% v/v HCl-washed, Milli-Q rinsed), it was rinsed with 5 ml Milli-Q to ensure all Se remained in the sample. Analysis of the sample proceeded as per Se(IV) determination.

2.3.3 Results and discussion

Cryogenic trapping is already being utilised in this method, so for additional improvement of the limit of detection co-precipitation was the most applicable of the preconcentration methods, when the analytical method and desirability of shipboard use were considered. Batch co-precipitation was tested for efficiency at stripping Se(IV) from the sample. Use of a 200 ml sample allowed for a 20-fold preconcentration. Samples used were 0.7 M NaCl and aged deep seawater with Se additions (0.063 nM and 0.63 nM), the samples were treated in 250 ml HDPE bottles. These bottles are routinely used for field sampling. The samples were spiked at the low Se concentration to mimic natural samples, and at the high Se concentration to determine if Se(IV) would be quantitatively recovered. Samples

were vacuum filtered to recover the precipitate. This was then dissolved in HCl to give a final volume and acidity matching those necessary for Se(IV) analysis.

2.3.3.1 Lanthanum concentration

Tests were carried out using various concentrations of lanthanum nitrate (2.5-10.0% w/v) (Table 2.6). All concentrations were seen to cause precipitation at elevated pH. Increasing concentration of lanthanum caused increased precipitation. Whilst this is not a problem for a batch method, if this work was adapted to an on-line method, higher levels of precipitation would make collection difficult due to the nature of knotted reactors [139].

Table 2.6 Recovery of selenium(IV) additions (0.063 and 0.63 nM) from different samples with varying lanthanum concentrations

Concentration of La added	0.7 M NaCl		Deep seawater	
	0.063 nM	0.63 nM	0.063 nM	0.63 nM
2.5% w/v	98.6%	94.6%	98.8%	92.4%
5% w/v	99.4%	100.3%	101.2%	98.9%
7.5% w/v	98.8%	99.4%	100.1%	97.5%
10% w/v	98.5%	101.7%	99.6%	102.2%

There was incomplete recovery of the added Se when 2.5% w/v La was used. All the other concentrations tested showed good recovery of Se(IV). 5% w/v La was chosen as the optimum as it gave good recovery whilst minimizing addition of reagents.

2.3.3.2 pH of precipitation

The volume of addition of ammonia/ammonium hydroxide was investigated for the precipitation of lanthanum hydroxide and Se. The amount added needed to raise the pH to over 9 to ensure complete precipitation of Se(IV) [144], even

though $La(OH)_3$ begins precipitating at pH~7.8 [145]. Raising the pH further (i.e. pH~12) did not increase the recovery of Se from the sample. Therefore, the minimum amount of ammonia/ammonium hydroxide was added to achieve the appropriate pH. The filtrate solution was analysed for Se(IV) to see if some remained in solution after precipitation. Se(IV) was undetected in the filtrate, even at 0.63 nM Se(IV) addition.

Unspiked NaCl solution was used to measure contamination from the reagents used in the co-precipitation step. No Se(IV) was detected to come from these reagents.

2.3.3.3 Selenium(VI)

Samples were spiked with Se(VI) (at 0.63 nM) to determine if it was collected by the precipitate. The dissolved precipitate was subjected to the HCl/KBr reduction, as was the filtrate, to determine where the Se(VI) was. Se(VI) was seen to be in the filtrate, it did not appear to be precipitated under the conditions tested.

2.3.4 Conclusions

Co-precipitation is a valid method for Se(IV) preconcentration. Se(IV) is the biologically relevant species and is expected to have levels close to the detection limit of the HG-CT-AFS method in open ocean surface waters with high biological activity. This co-precipitation method has been shown to be capable of a 20-fold preconcentration and could be applied readily to the developed analytical method for Se determination.

2.4 Conclusions

The method developed here has the capability to determine Se species at levels seen in the open ocean waters. The various steps necessary for determination of Se species have been tested and the method found to be suitable. Investigation into preconcentration, in case of lower than expected levels of Se, has shown co-precipitation to be valid for use with Se(IV). This method will now be applied to open ocean samples.

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Chapter 3

The latitudinal distribution of selenium in the Southern Ocean

3.1 Introduction

3.1.1 Study region: physical oceanography

The Southern Ocean covers water masses ranging from subtropical to polar, with division into broad oceanic regions separated by frontal zones (Figure 3.1), which can be identified by strong surface temperature and salinity gradients. To the north, the Subtropical Front (STF), found ~38–41°S, divides the warm salty waters of the subtropical region from Southern Ocean waters and the eastward flow of the Antarctic Circumpolar Current (ACC) [1]. The STF has been identified as a single front but also more broadly as the Subtropical Frontal Zone, covering 4–5° latitude which may have several fronts separating relatively similar waters [2].

South of this frontal region is the circumpolar ACC, which connects the major ocean basins. It has three frontal features. To the north is the Subantarctic Zone (SAZ), bound by the STF and to the south by the Subantarctic Front (SAF). In the Southeast Indian Ocean region it is situated at approximately 46–49°S, as it lies just north of the Southeast Indian Ridge [3]. The Polar Front (PF), the southern boundary of the Polar Frontal Zone (PFZ), is quite close to the SAF in this region and coincides with the Southeast Indian Ridge at ~51°S (Figure 3.2). The PF has been identified as two separate fronts, the Northern Polar Front and the Southern Polar Front [4], spanning the Antarctic Zone (AZ), which extends south to the Southern ACC Front (SACCF), which is the southernmost front of the ACC. South of this front is the Southern Zone [5], which contains the southern boundary of the ACC (Figure 3.1).

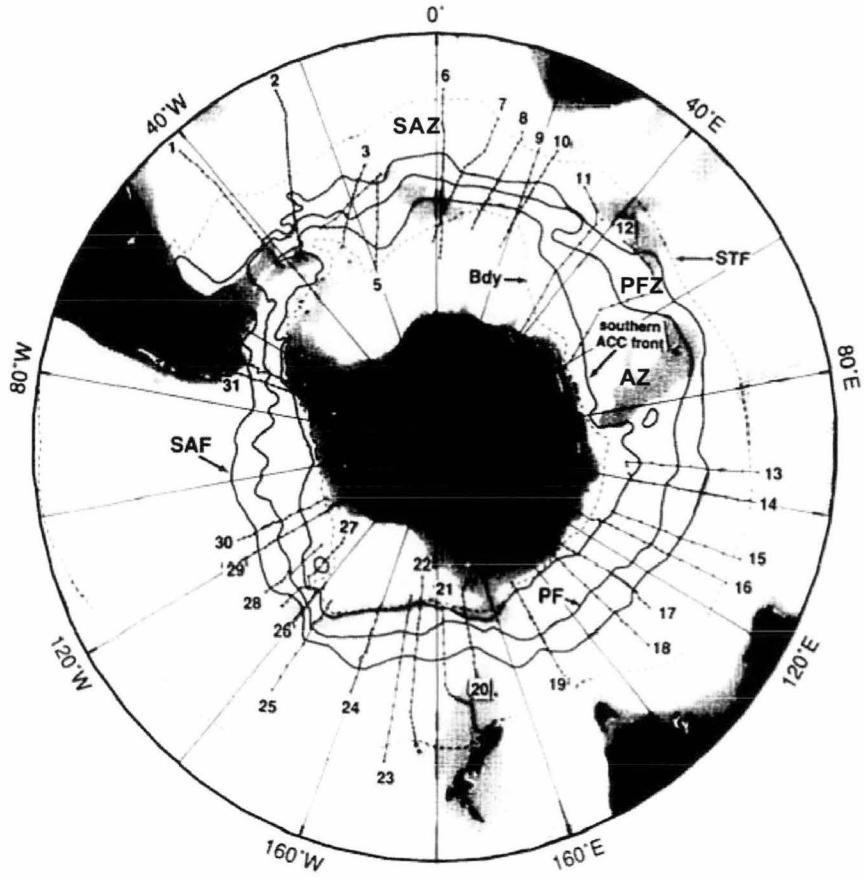

Figure 3.1 Front positions in the Southern Ocean. The Subtropical Front (STF) is marked by the dashed line; solid lines mark the Subantarctic Front (SAF), Polar Front (PF), and Southern ACC Front (SACCF); a dashed line marks the Southern Boundary of the ACC (Bdy). The different zones of the region are marked – Subantarctic Zone (SAZ), Polar Frontal Zone (PFZ), and Antarctic Zone (AZ). Image taken from Orsi et al., 1995 [1]

The Southern Ocean is a high nutrient-low chlorophyll (HNLC) region, with the wind-driven ACC causing the shoaling of nutrient-rich deep waters towards the south [6]. The nutrients, nitrate and phosphate, increase sharply in the PFZ, with silicate concentrations increasing further to the south in the AZ.

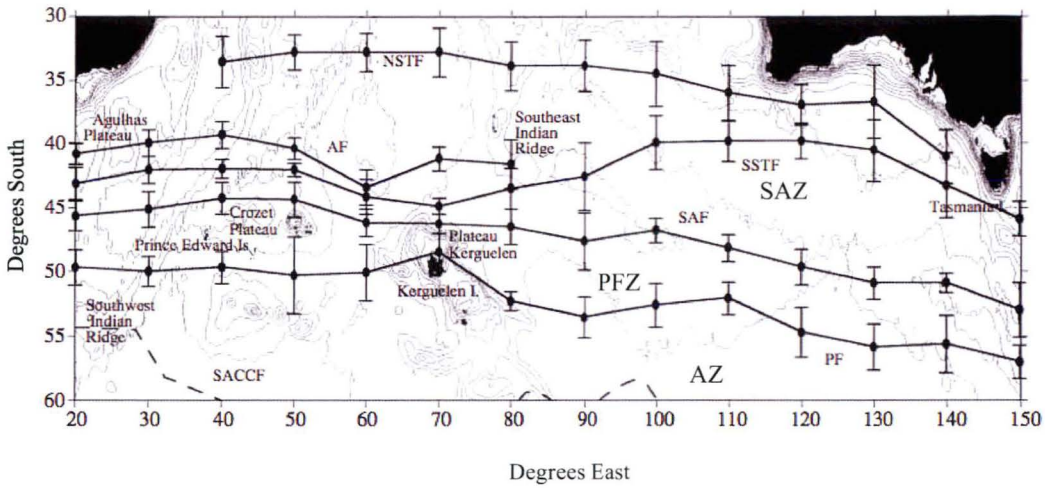

Figure 3.2 Frontal locations in the South Indian Ocean. The fronts are the Northern and Southern Subtropical fronts (respectively NSTF and SSTF); the Agulhas Front (AF); Subantarctic Front (SAF); and Polar Front (PF). The dashed line shows the location of the Southern Antarctic Circumpolar Current Front (SACCF). The zones of the region are the Subantarctic Zone (SAZ), Polar Frontal Zone (PFZ), and Antarctic Zone (AZ). Image taken from Kostianoy et al, 2003 [7]

Chlorophyll *a* (chl *a*) concentrations vary seasonally and spatially but the general trend in the Southern Ocean is for higher levels in the SAZ, decreasing to lowest levels in the PFZ and then increasing again in the AZ, with higher levels on the Antarctic coastal shelf and in the melting sea-ice region in spring [8-11]. The changes in nutrient concentrations and chl *a* are reflected in the structure of the phytoplankton communities. The frontal zones act as boundaries for geographic variations of phytoplankton size classes and functional groups [12, 13], with coccolithophores only found north of the SAF and a greater proportion of diatoms found south of the PF [14], in waters containing higher silicate concentrations.

As discussed in Chapter 1, the low productivity of the Southern Ocean is not a result of macronutrient limitation (N, P and Si) but other limiting factors – possibly low light, low iron, deep mixing or a combination of factors. Iron fertilisation, both natural and artificial, has been shown to positively influence productivity in HNLC regions [15], with various studies in the different regions of the Southern Ocean (e.g. [16, 17]). Other transition metal elements have also been

seen to be essential to microalgal species composition. They function as cofactors in metalloenzymes and proteins or vitamins, and those identified include Zn, Cu, Ni, Cd, Co, Mn and Mo [18]. These elements have different roles in the cells, but for some elements, for example Zn, which is essential to select phytoplankton, the cells can also substitute an element such as Cd or Co to meet the requirement [19, 20]. Oxyanions, including Se and V, have also been recognised to have important roles [21], but their exact biochemical function remains unclear [20].

The spatial and seasonal variation of the productivity of the Southern Ocean, combined with the infrequency of data collection (particularly for trace metal micronutrients), limits knowledge of all of its regions. The area of study for this work was the CLIVAR I9S line (~115°E) south of Western Australia, which was a repeat hydrography program. The I9S section was last occupied in 1994–1995. This approximate transect has also previously been studied 10 years earlier, in 1984 [22]. The line is close to the annual passage of the Japanese Antarctic Research Expedition (JARE) resupply vessels, which collect biological data, providing a historical record of productivity since 1965 [23].

3.1.2 Selenium in the ocean

The first measurements of Se in seawater reported very high concentrations. Due to recent improvements in sampling methods and the sensitivity of analytical techniques, we now know such data were incorrect. Work by Schutz and Turekian [24] in 1965 presented the first realistic oceanographic Se data as part of a series of wide-ranging analyses of oceanic waters for trace elements. Total Se concentrations (0.66–1.39 nM) were determined in samples from off Antarctica, the East Pacific and Atlantic Oceans, including the Caribbean Sea. More reports of Se measurements in seawater began to appear in 1976, when Sugimura et al. [25] reported total Se, and for the first time Se(IV) and Se(VI) concentrations in seawater, from the western North Pacific Ocean. Total Se ranged from 0.76–1.52 nM in the surface waters, remaining uniform throughout the mixed layer and was seen to increase with depth (up to a maximum of 2.53 nM). Se(IV) was observed throughout the water column, with lowest concentrations in the surface mixed layer (0.63–0.76 nM), increasing slightly below this and then remaining

constant to depth (0.76–1.14 nM). Se(VI) also showed lower concentrations than Se(IV) in the surface mixed layer (0.25–0.38 nM), with increases in deep water to the same levels as seen for Se(IV).

Measures and Burton [26] first linked inorganic Se species to biological processes. Their work noted the nutrient-like profiles of the inorganic Se species — exemplified by the correlation between Se and macronutrients: Se(IV) with silicate, and Se(VI) with phosphate (or to be precise, with phosphate and silicate, depending on sample location).

The general trend reported for the inorganic Se species in the ocean is depletion in the surface waters and increases in the region of oxygen minimum [26]. The concentration into deeper water then varies only slightly. Concentrations in surface waters can be extremely low: Se(IV) below 0.025 nM, and total Se between 0.43 and 0.65 nM [26] (Table 3.1). Changes from these expected profiles have been reported, but are explained by the presence of various hydrographic features (e.g. Amazon River plume [27], or different origins and history of water masses [28]). Measures et al. [29] compared Se values from the three ocean basins (all 500+ measurements were made with the same analytical method), finding that whilst all ocean basins displayed similar profiles for Se(IV) and Se(VI), the Atlantic Ocean had total Se concentrations 30-40% lower than the Pacific and Indian Oceans. This represents a good example of trace element inter-ocean fractionation of a nutrient-like element, due to the global thermohaline circulation. The residence time of inorganic Se has been estimated in deep waters, with a mean for residence time for Se(VI), with respect to the oxidation of Se(IV), of 1500–2000 yr [30].

In 1979, a new form of Se was defined in seawater measurements. The analytically defined 'organic Se' was first measured by Suzuki and colleagues [31]. Organic Se has not been directly determined and is always calculated by difference from total Se and inorganic Se after treatment (see Chapter 2, section 2.2.1.2 – Total selenium determination). Methods for Se determination vary and there are different ideas on the abundance of organic Se in the open ocean. Organic Se has been seen in high but variable concentrations (a result of

biological and nutrient status of specific sites) in surface waters, but there is some disagreement as to whether organic Se is a measure of organically bound Se or an artefact of the analytical method. In some studies, organic Se was considered to contribute negligibly to the total Se pool in the open ocean, so Se(VI) was determined from total Se minus Se(IV), with organic forms of Se included in this measurement [26, 32]. Other studies use various methods to distinguish the three defined[1] oceanic Se species (Se(IV), Se(VI) and organic Se). From these studies, organic Se is thought to be dominant in the surface waters in some areas of the ocean (e.g., greater than 85% of total Se in the high latitude north Atlantic Ocean [28], and 50±11% of total Se in the equatorial Atlantic Ocean [27]). However, in the mesotrophic waters of the far north Atlantic Ocean, levels of organic Se were low, which was attributed to relatively low nutrient availability, and thus, intermediate levels of productivity of the region [28].

3.1.3 Selenium in the Southern Hemisphere oceans

Oceanic studies of Se have mainly focused on the Northern Hemisphere, and most information on Se distribution has been gained from the Pacific and Atlantic Oceans. There is a lack of information on Se in the Southern Hemisphere marine environment. Very few studies have been carried out, with only a handful of isolated stations providing incomplete information on Se in relation to basin-scale oceanography. This makes it difficult to draw conclusions on the oceanographic behaviour and biogeochemistry of Se.

Sherrard et al. [34] investigated seasonal Se speciation and phytoplankton interactions in subtropical and subantarctic waters to the east of New Zealand. They found inorganic Se concentrations dominated the total Se pool for the majority of the year, although organic Se persisted in surface waters (average values 0.008-0.11 nM). The biological utilisation of Se was linked to the observed nutrient-like profiles as previously reported. For example, in the subtropical waters, decreases in Se(IV) (the preferred species for biological uptake) and increases in organic Se were linked to observed spring phytoplankton blooms.

[1] This definition of the different Se species is an methodological classification, which has become common practice [33].

However, in the subantarctic waters, Se speciation trends were found to be relatively constant, regardless of phytoplankton seasonal variability.

The eastern Indian Ocean was sampled at four stations, from 10°S to 40°S, with Se measured and compared to macronutrients, temperature and salinity [35, 36]. This work reported organic Se to be the dominant Se species at 3 stations at all depths, which is contradictory to other published work. The methods used for speciation in this study appear to be inconsistent (possibly due to incomplete conversion of Se(VI) by the heated acid bromide digest[2]), resulting in elevated organic Se values and reduced values for Se(VI), since organic Se is determined by subtraction of total inorganic Se from total Se. If only Se(IV) and total Se data is examined in this study, the trends are similar for those reported in other oceans. A nutrient-like profile was evident for Se(IV) with very low euphotic layer concentrations (0.02–0.12 nM), in the range of 0.5 nM to 0.7 nM at 1000 m, and with gradual increases to depth attaining a maximum concentration of 0.79 nM below 2000 m. Se(IV) profiles were similar across all stations. Total Se was lowest in surface waters, and the concentrations at the northern stations were lower and displayed a slightly different profile. The northern stations had ~0.5 nM total Se at the surface, with a rapid increase to a maximum at 1000 m. The southern stations had ~0.7 nM total Se at the surface with minimal increase until 1000 m, and a maximum at 2000 m.

There have been a limited number of Se measurements in Antarctic waters. Papoff and colleagues [37, 38] used an electrochemical method to measure Se at two sites in the Ross Sea, one coastal and one open water. Their study showed Se(IV) and total inorganic Se to increase with depth, as has been seen in other oceans. Organic Se in the surface water was 0.43 nM, with very low levels of Se(IV) (0.067 nM). This study was simply an application of a newly-developed analytical method, with no linking of the Se data to other oceanic information.

[2] The use of a heated acid digestion for the conversion of Se(VI) to Se(IV) was investigated and discussed in Chapter 2. The time between the reduction step and analysis was shown to affect the recovery of Se(VI).

Another study in the Antarctic (Prydz Bay, Indian Ocean sector) measured Se in 29 depth profiles [39]. Total Se in the surface water was 1.31 nM [39]. Throughout the water column inorganic Se accounted for 72% of total Se. The maximum value of Se(IV) (0.35 nM) was seen in the oxic zone below the mixed layer, with low levels at the surface. Se(VI) was dominant throughout the water column with small increases with depth. Organic Se had a maximum of 0.42 nM at the surface and was not seen below 1000 m.

Generally, Se(IV) is depleted in surface waters, with rapid increase in the oxygen minimum zone and a gradual increase to depth. Se(VI) follows the same trend but with less depletion in the surface waters and typically higher concentrations throughout the water column. Organic Se is only expected to be found in the surface waters of the ocean [40], although in suboxic and anoxic regions, where it may be stabilised (by isolation from oxidative processes of mineralization), it will persist and its concentration may increase [41]. The distribution of inorganic Se species will also be influenced by changes in redox potential in low oxygen water masses.

This study provides information on the concentration and distribution of Se (and its different species) in the Indian sector of the Southern Ocean. The data is compared to other oceanic parameters—physical, chemical and biological—to give a greater understanding of the biogeochemical behaviour of Se.

3.2 Methods

3.2.1 Study area and sampling

Seawater samples were collected on a voyage of the RSV *Aurora Australis* (au0403, V3 2004/2005, 'ORCKA') in the Southern Ocean during the austral summer 2004-2005 (December-January). The cruise reoccupied the WOCE CLIVAR I9S line (approximately 115°E). A map of the region and the sampling locations are shown in Figure 3.3. Samples were collected from the full depth of the water column and in each of the oceanic zones, covering all Southern Ocean water masses from subtropical to polar, as described in Section 3.1.1. Sampling

took place over a 3-week period so the effect of seasonal variation on samples should be minimal.

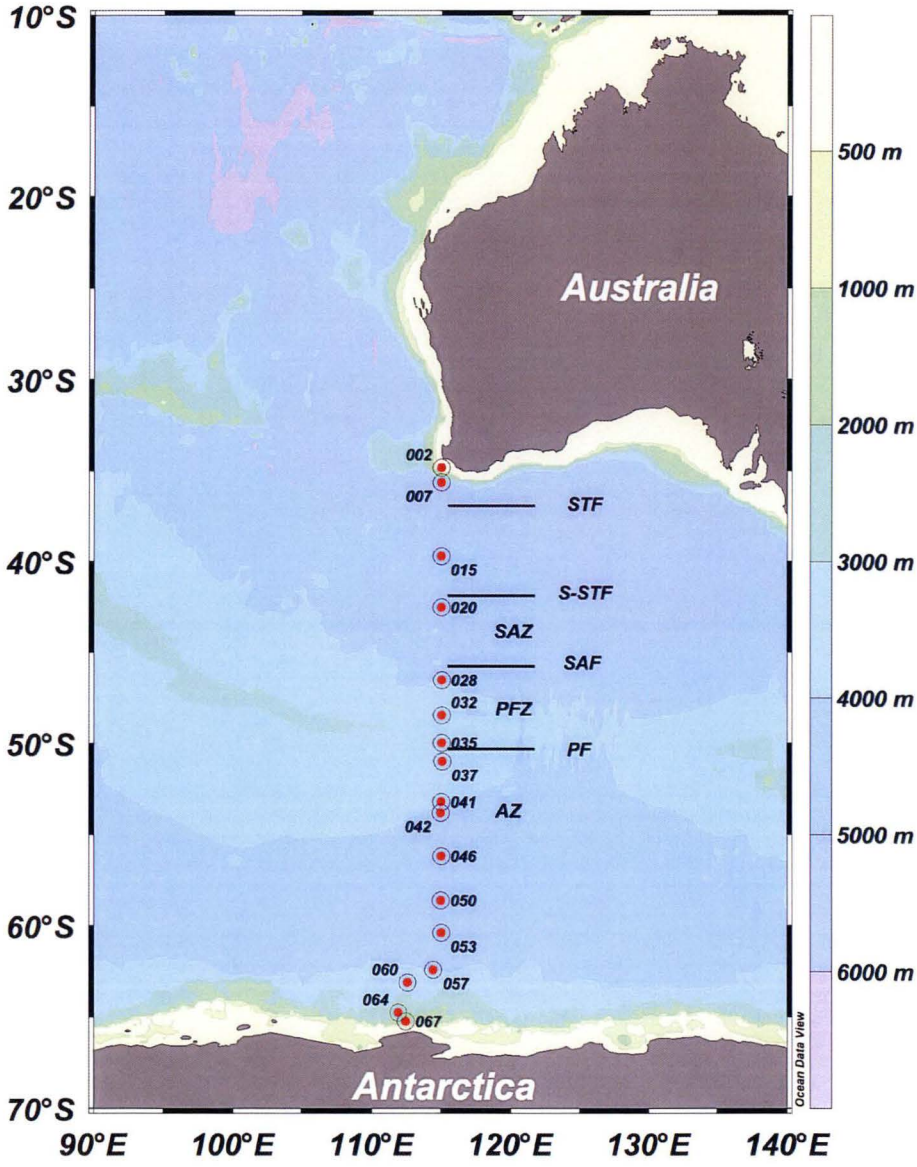

Figure 3.3 Map showing station locations in the Southern Ocean, from the southwest of Australia at the north to Antarctica at the south, where samples were collected for selenium analysis along the CLIVAR I9S transect. Fronts and zones are indicated (prepared using Ocean Data View [42])

Water samples were collected using 10-l Niskin bottles (General Oceanics, Florida, USA) deployed on a standard conductivity-temperature-depth (CTD) rosette. There was no special cleaning of the Niskin bottles, although the rosette was wiped with acetone between casts (acetone cleaning was done at the request of the CFC samplers). Samples were subsampled from the Niskin bottles into acid-cleaned 250-ml HDPE bottles that were rinsed twice with sample before filling. The 250-ml samples were acidified with 750 µl of HCl (Mallinckrodt Baker Inc., Paris, Kentucky, USA; 12 M) to pH 1.6 and shaken well. Samples were analysed for Se(IV) within one week of sampling. Trace metal clean handling procedures, including wearing of polythene gloves, were followed, wherever possible.

3.2.2 Analysis of samples

Samples were analysed onboard the ship for Se(IV) by hydride generation–cryogenic trapping–atomic fluorescence spectrometry (HG-CT-AFS). Due to time constraints, all Se(VI) and total Se measurements were conducted on return to the shore-based laboratory.

The analytical method used is described in Chapter 2 (section 2.2.2.3) and published in Wake et al. (2004) [43]. Shipboard modifications necessary for operation at sea are described in Chapter 2, section 2.2.2.4.

The HG-CT-AFS instrumentation has previously been used at sea for As analysis [44], but this work represented the first use of the modified system for at-sea Se analysis. To ensure consistency in the performance of the system, an aged deep seawater sample was routinely analysed (at beginning of run and then approximately every eight samples) as a charting substandard.

3.3 Results and discussion

3.3.1 Nitrite interference

Interference by nitrite on the Se method was removed by the addition of sulfanilimide to an acidified sample before analysis (see Chapter 2; but only valid up to nitrite concentrations of 4 μM). Nitrite was not measured routinely on the voyage, but full depth profile samples were collected and measured onboard on request to confirm that nitrite would not be above the threshold concentration for interference. Nitrite levels were highest in the surface waters, but only reached a maximum value of 0.22 μM. Below 140 m depth, nitrite was below the limit of detection (< 0.1 μM). This confirmed previous reports of low nitrite concentrations in the Southern Ocean (reported for the Atlantic sector – maximum concentrations of <0.4 μM [45]), and ensured that it would not be expected to interfere with Se determinations.

3.3.2 Stability of samples

Shipboard analysis of samples prevents the need for storage. However, in this work, not all Se species were analysed on-board ship; and therefore, a stability study was undertaken during this voyage to ensure that results were consistent and accurate. Se(IV) samples were analysed onboard, but time between collection and analysis varied from 1 to 7 days. Acidification has been shown to preserve speciation, but Cutter [46] reported that acidification was unnecessary up to two weeks after collection. Cheam and Agemian [47] report that samples, spiked with inorganic Se, at 12.7 and 126.6 nM, were stable unacidified , over a 4 month study, in polyethylene bottles if stored at 4°C in the dark. Acidification at high acid levels can cause species interconversion [46, 48], so samples were acidified to pH~1.6, as is common in the literature [30, 49-52]. Acidification to pH 1.5 is reported to remove the effects of the container type and stabilize the sample no matter what the matrix is [47]. Se(VI) is believed to be more stable than Se(IV) [47, 53, 54], but its exact stability is unknown.

Representative samples were collected from surface, mixed layer and deep waters. Deep samples are stated to be more stable than surface samples because dissolved oxygen, which in general is higher at the surface, speeds the oxidation of Se(IV) to Se(VI) in laboratory experiments [55]. No reports were found in the literature of open ocean samples being stored unfiltered and acidified for longer than three months. The conditions of this work varied from previous reported studies in container material (polyethylene instead of glass [27, 30]) and length of storage. Sugimura and Suzuki [56] report open ocean unfiltered samples, acidified to pH~2, to be stable for at least 5 weeks in polyethylene bottles. The samples were left at natural pH or acidified with HCl (3 ml per litre of sample, as per [27]); all other storage conditions were the same. Analysis for Se(IV) was done within 24 h of collection and then at 3, 7 and 14 days. Se(VI) analysis was conducted at 1 and 7 days. The samples were again analysed on the return to the shore-based lab (~2 months and ~6 months after collection). Samples were found to be stable, under the conditions tested, for Se(IV) and Se(VI), with differences within the confidence limits of the methods (data not shown).

Analysis for total Se was not possible at sea, and the stability of this component is unknown. Most organic Se stability studies are concerned with the volatile species (not measured in this study), which are reported to be stable for only 24 hours [53]. There have been studies into the stability of various Se-amino acids complexes, but at elevated concentrations (e.g. 316 nM and 1.90 μM in a freshwater matrix [57]). It is reported that Se-methionine is more stable than either of the inorganic Se species, Se(IV) or Se(VI) [58]. Sample acidification is standard practice for storage of samples for later measurement of the three Se species [27, 34]. No previous reports were found investigating the effect of storage of seawater samples under these conditions.

3.3.3 Oceanographic conditions

The voyage crossed a number of fronts, with some delineation between individual features (Figures 3.4 and 3.5). The presence of multiple fronts (e.g. the southern PF) has been seen repeatedly in the Australian sector of the Southern Ocean [2]. Nutrient concentrations increased southward as expected and there was a shallowing and weakening of the oxygen minimum zone from north to south (Figure 3.4). All supporting data for Se sampled stations can be found in Appendix 1, with the complete hydrographic data set in reference [59], also available online at http://data.aad.gov.au/aadc/metadata/.

There were two distinct STF, as is typically seen in this region, the northern-STF was located at ~37°S and the southern-STF at ~42°S. The SAF was ~46°S and the PF at ~50°S. There were multiple SACCF seen during this voyage.

Near the Antarctic continent, northward flowing Antarctic Bottom Water (AABW) dominates the deep water masses, identified by low salinity and temperature (below 0°C) [60] (Figure 3.4a). Lower Circumpolar Deep Water (LCDW) is seen throughout the transect in the mid-ocean, identified by higher salinity; it shallows from ~3000 m at the northern end of the transect to ~1200 m around 60°S. Antarctic Surface Water (AASW) is seen in the upper 300 m in the North Polar Frontal region, whilst Antarctic Intermediate Water (AAIW) is seen at the northern end of the transect at ~1000 m. At the southern end of the transect, there was increasing sea-ice cover, with the final station in heavy sea-ice.

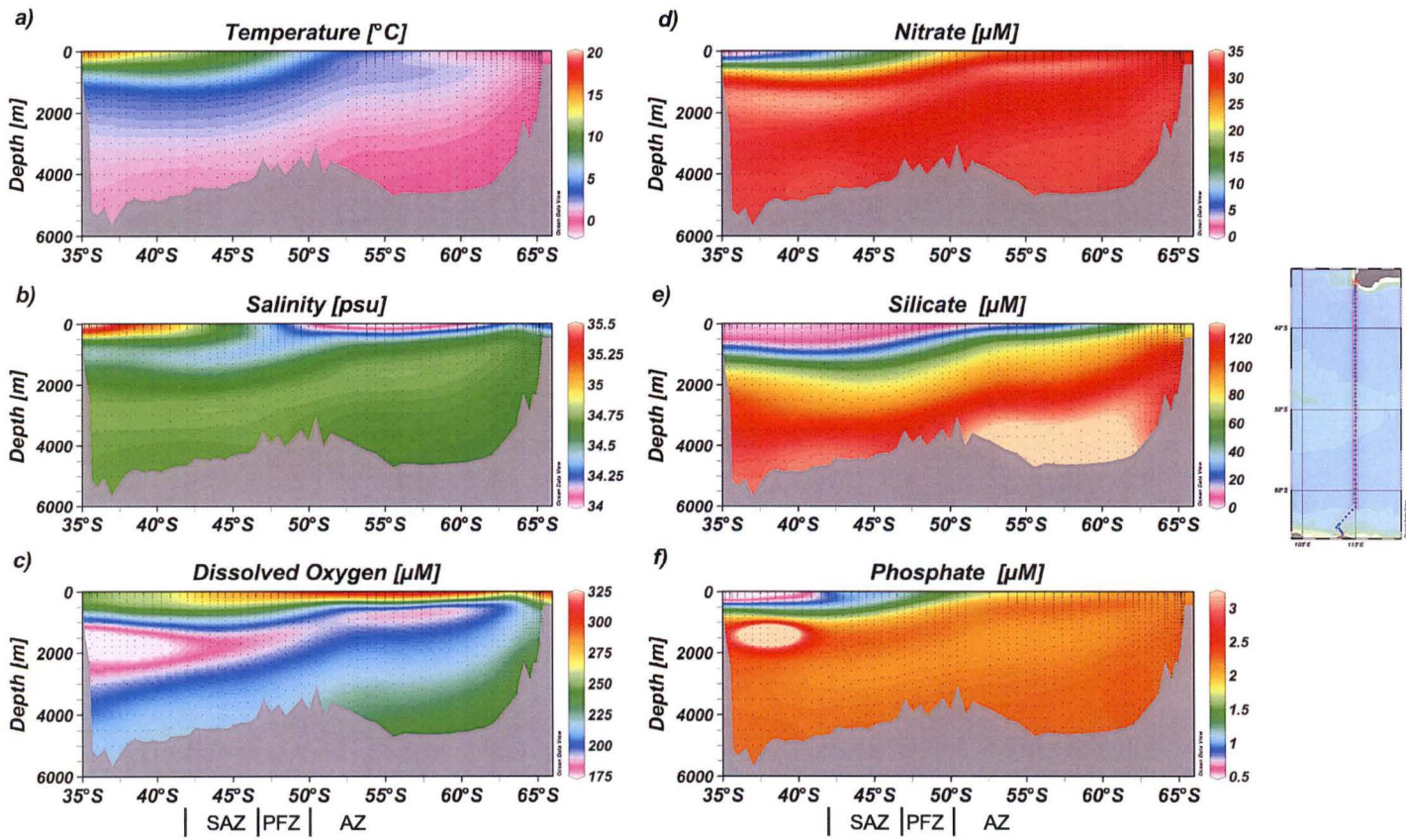

Figure 3.4 Properties of the full water column along the CLIVAR I9S transect a) temperature b) salinity c) dissolved oxygen d) nitrate e) silicate f) phosphate

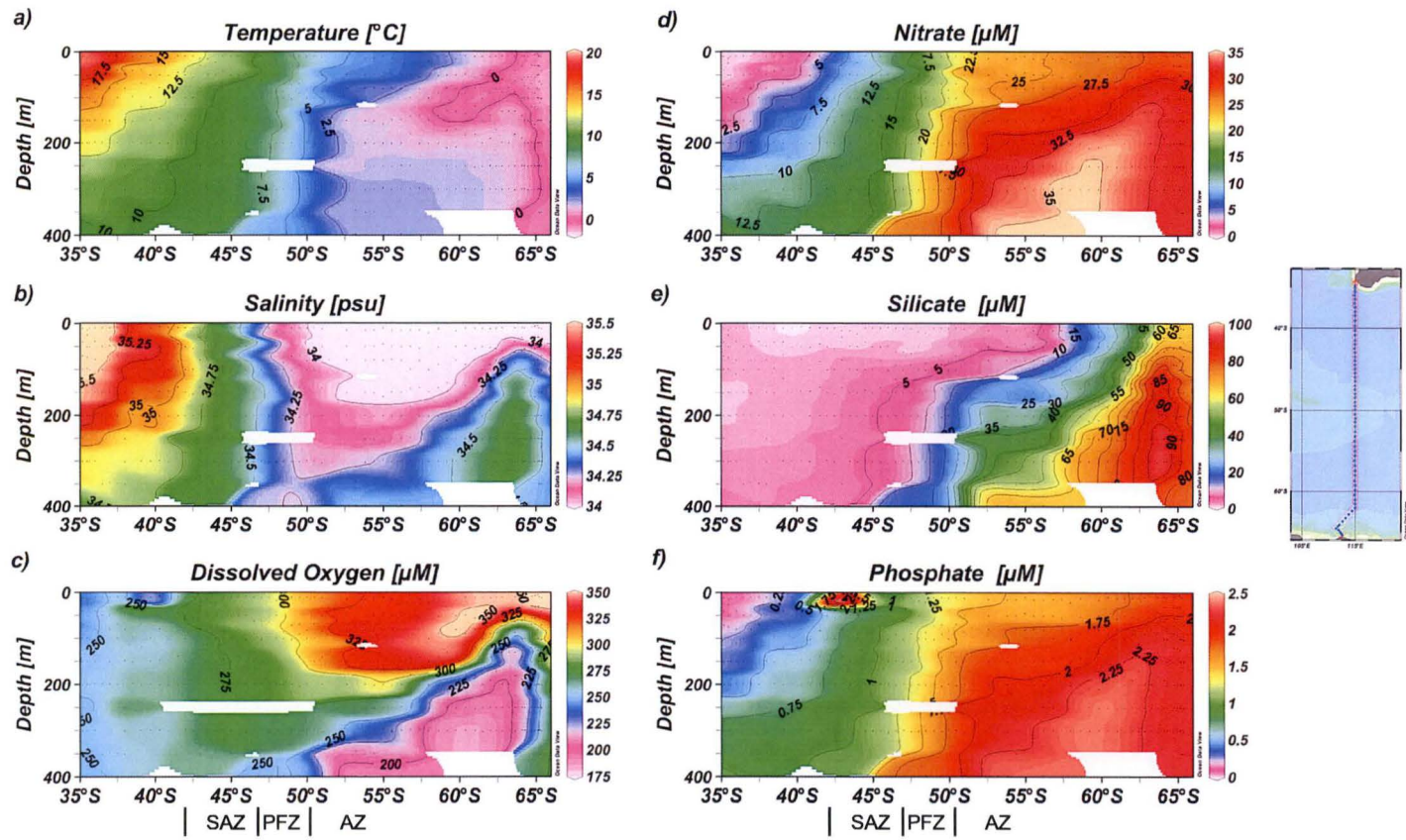

Figure 3.5 Properties of the upper 400 m along the CLIVAR I9S transect a) temperature b) salinity c) dissolved oxygen d) nitrate e) silicate f) phosphate. NB: different scale on Z-axis from previous figure

3.3.4 Biological information

The phytoplankton biomass of the region can be seen from the satellite image of chl *a* (Figure 3.6) and the chl *a* data for the voyage (Figure 3.7). The satellite image shows higher surface chl *a* at the northern end of the transect and this is confirmed by the measured chl *a*. Higher levels of chl *a* biomass were also observed between ~37–47°S, corresponding to the SAZ, which is known to be a productive region of the Southern Ocean. There is again an increase south of 62°S, where the highest levels of chl *a* were seen on the transect (Figure 3.7). This was not seen on the satellite image, possibly because of sea-ice or cloud cover.

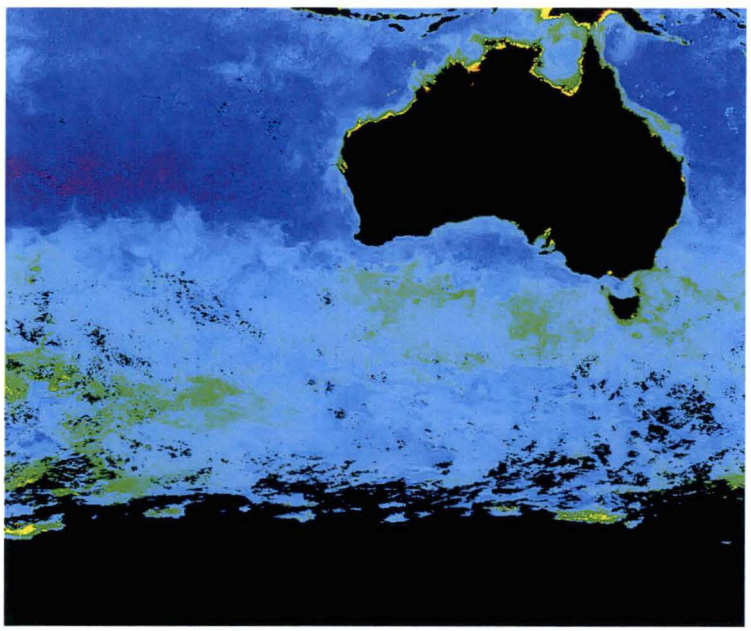

Figure 3.6 Monthly composite satellite image of chlorophyll *a* concentrations in the Australian sector of the Southern Ocean for December 2004. Provided by the SeaWIFS Project, NASA/Goddard Space Flight Center and GeoEye (http://oceancolor.gsfc.nasa.gov/cgi/browse.pl – accessed 10 August 2009)

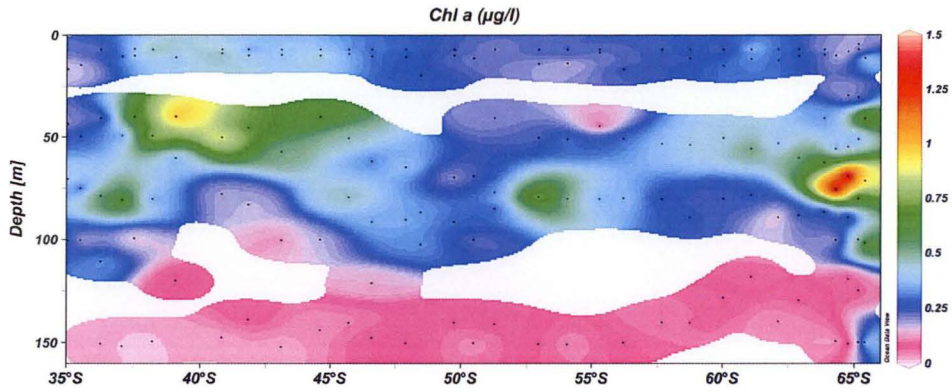

Figure 3.7 Chlorophyll *a* concentrations measured in the surface waters (upper 160 m) along the CLIVAR I9S transect (data courtesy of S. Wright, Australian Antarctic Division)

The biological data for this region has been logged over many years, as the I9S line is close to the route of the JARE resupply ship. This, along with data from other biological surveys in the region, helps discern the trends in phytoplankton speciation [23, 61]. Typically for this region, the contribution of the different phytoplankton species to chl *a* is dominated by diatoms in the polar waters; with dinoflagellates contributing in the SAZ [61]. Diatom growth would be limited north of the Polar Front, because of the depletion of silicate in the surface waters (Figure 3.5e).

3.3.5 Surface transect

All Se species showed a general increase in surface water concentrations from lower to higher latitudes (35–65°S) (Figure 3.8). This general latitudinal trend of higher Se concentration at higher latitudes has been noted previously by Weiping et al [39], who compared their data (at 63–68°S) to values measured in the equatorial Pacific (1.31 nM compared to 1.09 nM). Other than this comparison, there has been a lack of high latitude measurements, making it difficult to discern what concentrations of Se would be expected in the high latitude Southern Ocean. A summary of surface ocean Se concentrations is presented in Table 3.1.

Table 3.1 Surface water selenium concentration from the world's oceans

Region	Se(IV) (nM)	Se(VI) (nM)	Total Se (nM)	Ref.
North Atlantic	0.14±0.05	0.21±0.07	0.49±0.08	[28]
Atlantic Ocean				
– Northern Hemisphere			0.58±0.06	[51]
– Southern Hemisphere			0.56±0.07	
Atlantic Ocean	<0.025	0.713	0.738	[26]
North Pacific			0.50–0.80	[62]
North Pacific				[63]
– western (depth profile)	0.027	0.31		
– east to west transect	0.05–0.40	0.46		
Western North Pacific	0.50–1.01	0.13–0.76	0.76–1.52	[25]
Western North Pacific	0.53	0.35	1.05	[31]
South Pacific				
– Subtropical water	0.10–0.19	0.29–0.56	0.61–0.83	[34]
– Subantarctic surface water	0.14–0.24	0.51–0.69	0.76–0.99	
Southern Ocean, south of 60°S			1.31^	[39]
Southern Ocean				
– north of SAF*	0.09–0.17	0.32–0.43	0.51–0.64	This
– south of SAF	0.16–0.35	0.40–0.51	0.64–0.90	study#

^ average of 42 samples

* SAF located at ~46°S

for samples 0–50 m

The increase in Se(IV) with latitude was greater than that for Se(VI) (Table 3.1). The levels of organic Se were low (the highest level was 0.09 nM) throughout the surface waters of the transect (Figure 3.9). The highest levels for organic Se are seen north of 37°S, in the STZ, and between 45°S and 52°S, in the PFZ. The region of highest chl *a* biomass (Figure 3.7 and 3.9) lies between 37°S and 46°S; the biologically preferred Se(IV) was at the lowest levels in the centre of this region, 38°S to 43°S (Figure 3.8

and 3.9). The highest levels of organic Se were seen to the north and south of the large subsurface chl *a* maximum (Figure 3.9).

In summary, surface Se concentration increases southward, with a corresponding decrease in the temperature, salinity and chl *a* (not shown), and an increase in the nutrient concentrations (Figure 3.8). The relationship between the macronutrients and Se will be discussed further in section 3.3.9.

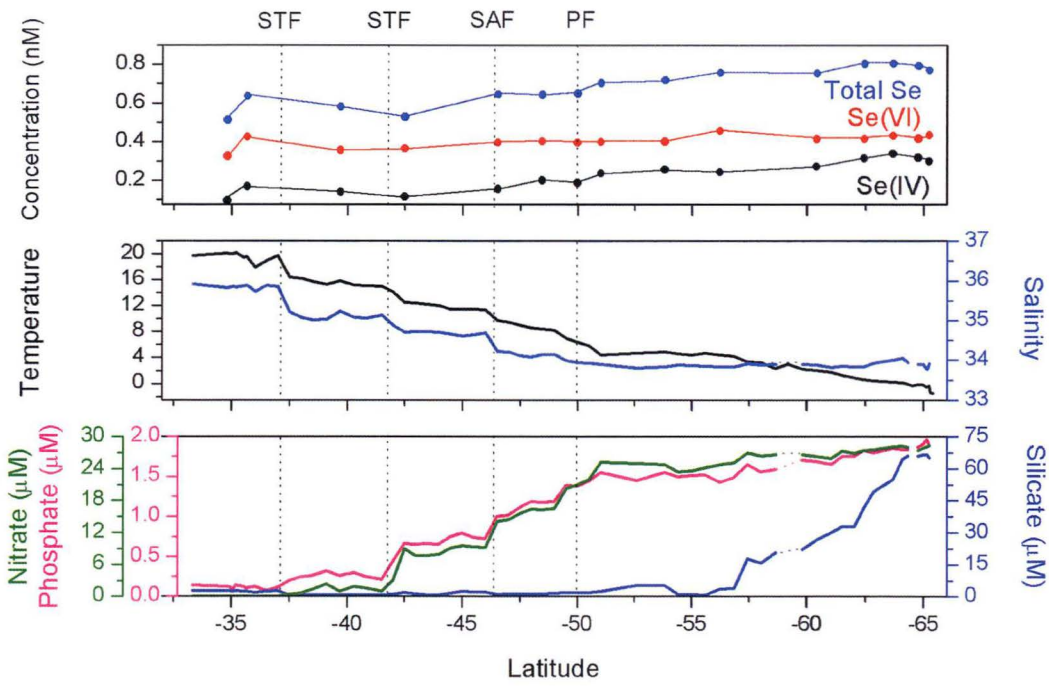

Figure 3.8 Selenium speciation, temperature, salinity and nutrient concentrations in surface samples (8–22 m) along the CLIVAR I9S transect

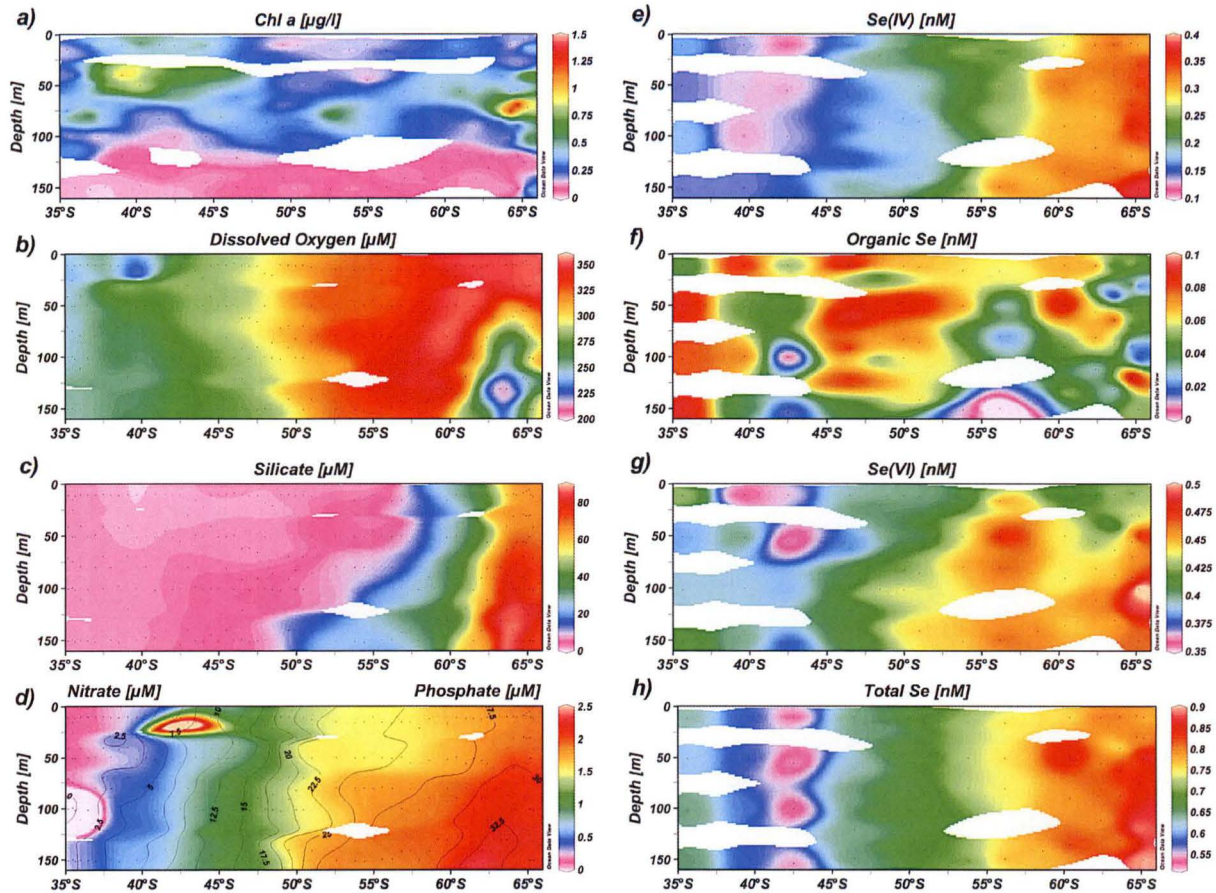

Figure 3.9 Properties of the upper 160 m along the CLIVAR I9S transect a) chlorophyll *a* b) dissolved oxygen c) silicate d) phosphate with nitrate overlaid e) Se(IV) f) organic Se g) Se(VI) h) total Se

3.3.6 Comparison with literature data

Comparison of the Se surface values (Se(IV), Se(VI) and total Se) for this study with those of previous studies show them to be consistent for open ocean regions (Table 3.1). Note, several differing opinions on the role of organic Se presently exist and whether organic Se is a by-product of biological productivity, so seasonality of studies may affect concentrations [34]. The nearest observations to our study area, the South Pacific to the east of New Zealand [34], showed similar concentrations for Se in subtropical water, but had higher levels of Se in subantarctic surface waters (Table 3.1), which were not seen in this study. The previous study examined water masses north and south of the Chatham Rise, which is a bathymetric feature causing very sharp gradients in water properties (note both subtropical and subantarctic surface waters for this study are listed in the Table 3.1 as north of the SAF). The organic Se values for that region (0.10-0.19 nM over the four seasons [34]) were similar to those seen in this study (Figure 3.9f, maximum value 0.09±0.04 nM), the exception was the value seen in subtropical waters in the summer, 0.31±0.04 nM [34](samples collected 18 January to 5 February 2000). The lack of Se data makes it difficult to directly compare the studies as seasonality and zonal trends (Pacific sector versus Indian sector of the Southern Ocean) could cause these differences. Further study is needed to clarify these ideas.

3.3.7 Vertical profiles

The vertical distribution of Se along the transect needs to be considered in the context of the model of global overturning circulation (Figure 3.10). The deep water masses shoal southward, these water masses transporting higher nutrient levels to the surface. The formation of new deep waters around the Antarctic continent causes the sinking of surface waters with higher oxygen levels and possibly biologically depleted nutrients.

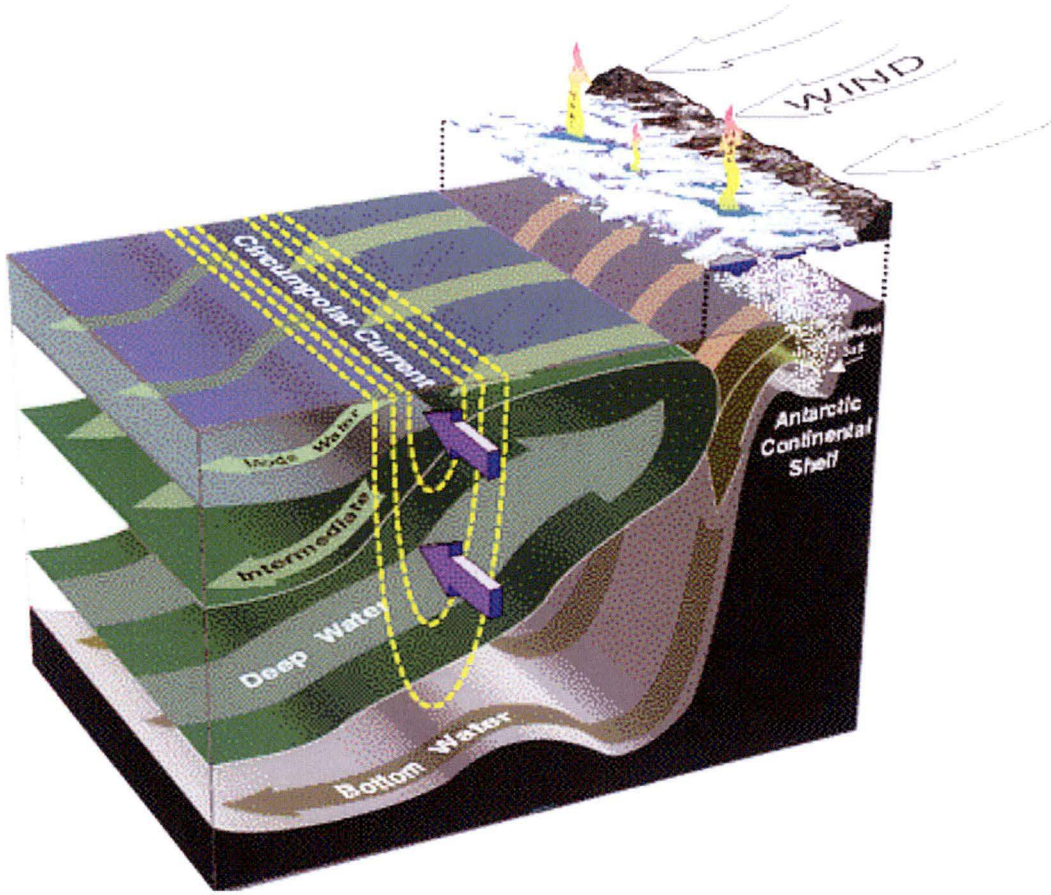

Figure 3.10 The global overturning circulation in the Southern Hemisphere http://www.cmar.csiro.au/news/media/archive/98releases/images/antarctic3d.gif - accessed 10 August 2009

Values for inorganic Se displayed nutrient-like profiles, as has been reported previously [26, 31-33, 63]. From low levels in the surface waters, there was an increase to the region of oxygen minimum (especially for Se(IV)) and then smaller variations with depth. As can be seen in Figure 3.11, the increase in Se concentrations southward seen in the surface waters continues throughout the full water column. Figure 3.12 illustrates the shallowing of the Se(IV) maximum southward.

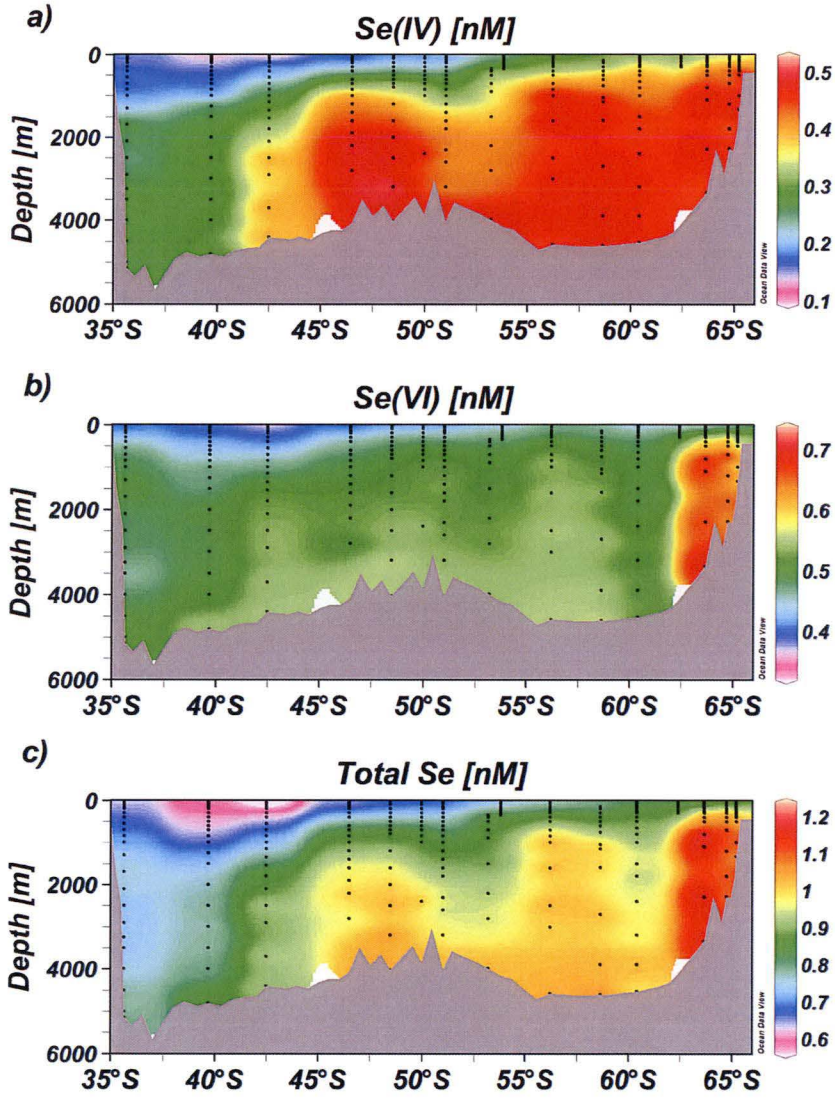

Figure 3.11 Full water column distribution of selenium along the CLIVAR I9S transect a) Se(IV) b) Se(VI) c) total Se

The maximum concentration of Se(IV) is seen to shallow southward (Figure 3.11a and 3.12). This change is more pronounced in Se(IV) species concentration (compared to Se(VI)) (Figure 3.12), perhaps reflecting the biological requirement of the reduced form, with higher productivity at the northern end of the transect causing a drawdown of this species. Smaller changes are seen in the concentration of Se(VI) with depth. This has been linked previously to the shallowing of the oxygen

minimum zone (Figure 3.4c for this cruise's oxygen data, Figure 3.13 for top 800 m of Se species and dissolved oxygen profiles), which is the area of maximum regeneration/remineralisation. The regeneration of organic material may be the reason for this increase in Se(IV), which is seen more strongly than in the Se(VI) profile.

A striking feature of the Se(VI) transect is the higher concentrations seen at the southern end of the transect, with deep water values consistent throughout the transect until this increase (Figure 3.11b). The cause of this increase is unknown – it may be a result of sea-ice formation and brine exclusion or a shelf sediment input.

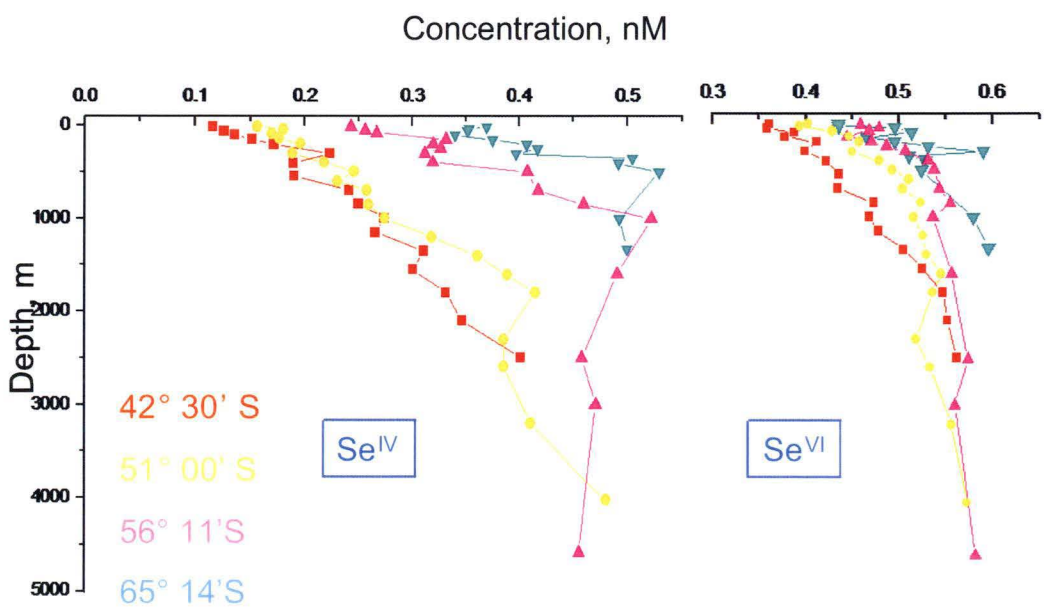

Figure 3.12 Depth profiles of selenium(IV) and selenium(VI) in different zones of the Southern Ocean

42° 30' S – SAZ

51° 00' S – AZ

56° 11' S – AZ

65° 14' S – Sea ice zone (SIZ)

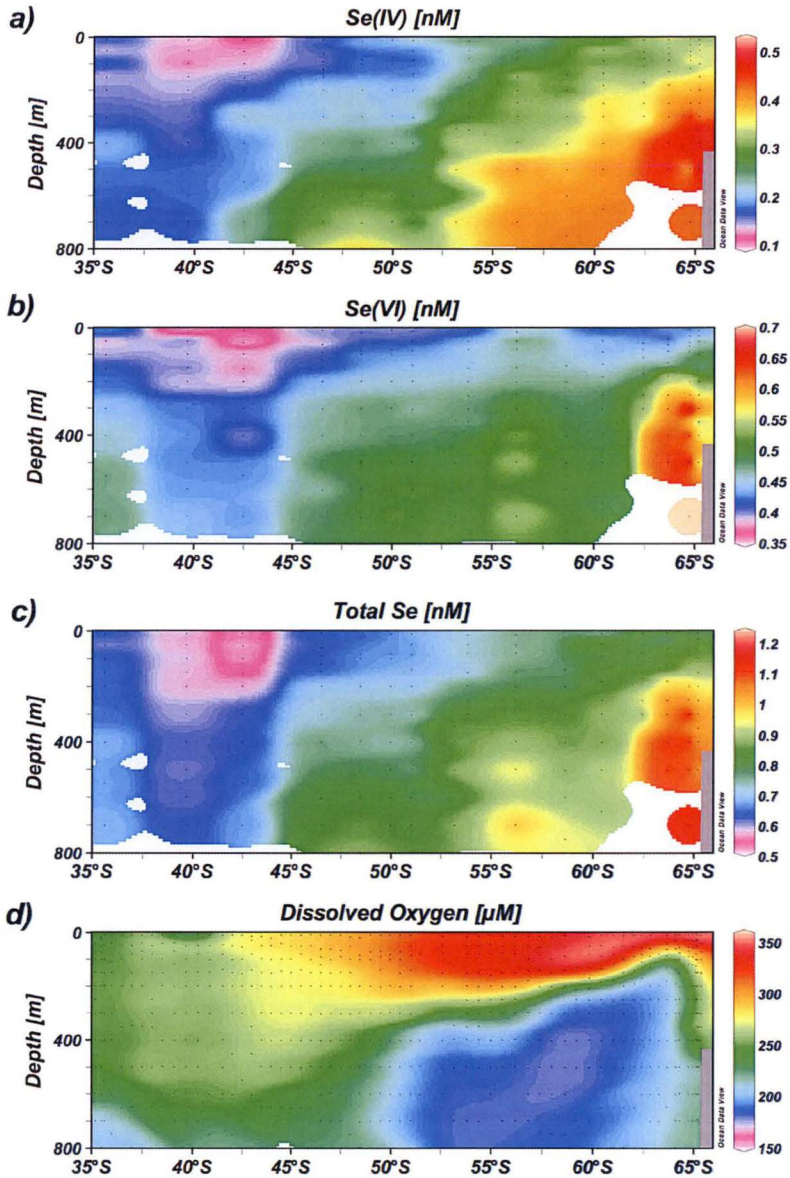

Figure 3.13 Properties of selenium and dissolved oxygen in the upper 800 m along the CLIVAR I9S transect a) Se(IV) b) Se(VI) c) total Se d) dissolved oxygen

3.3.8 Selenium(IV) to selenium(VI) ratios

The ratio of Se(IV) to Se(VI) has previously been reported ([34] and references within) and it is suggested that as a water mass ages, its Se(IV)/Se(VI) ratio should decrease as the concentration of Se(IV) decreases as it is slowly oxidised to Se(VI) [51]. Deep waters that are recently formed, such as those in the North Atlantic and regions of the Southern Ocean, would be expected to have a higher ratio relative to older deep water masses, such as in the North Pacific. These trends are not clear from the current available data (Table 3.2). Previously reported values are highly variable, even in the same oceanic region where the total Se concentrations are similar (see North Pacific data in Table 3.2).

The Se(IV)/Se(VI) ratio seen here is lower at the northern end of the transect (0.55±0.03 north of the STF), when the total Se concentration was also lower, and higher to the south (0.82±0.09 south of the STF (Table 3.2)). The Se(IV) to Se(VI) ratio seen north of the STF has similar total Se concentration and Se(IV) to Se(VI) ratio to one of the North Atlantic studies [28]. Cutter and Cutter [27] report that waters originating from the high latitudes of the Southern Hemisphere have a low Se(IV) to Se(VI) ratio, because of the enrichment of Se(VI) in these waters (reported ratio ~0.4). Whilst values this low were not seen in this study, at the southern most end of the transect, the ratio decreased to 0.68±0.04 (at 63.7°S, $n = 2$) from 0.88±0.03 (at 60.4°S, $n = 7$), as a result of the higher Se(VI) values seen close to the continent (Figure 3.11b).

Table 3.2 Total selenium concentration and ratio of Se(IV) to Se(VI) in deep waters

	Total Se (nM)	Se(IV)/Se(VI)	Reference
North Atlantic	0.77±0.04	0.58±0.18	[28]
	1.04±0.10	1.04±0.27	[51]
	1.20±0.10	0.65±0.06	
	1.41±0.18[a]	0.48±0.14[a]	[26]
South Atlantic	1.26±0.04	0.74±0.10	[51]
	1.19[#]	0.82[#]	
South Pacific	1.64±0.18[b]	0.71±0.03[c]	[34]
North Pacific	2.26±0.04[d]	0.55±0.02[d]	[30]
	2.24±0.14[e]	0.73±0.06[e]	[33]
Southern Ocean,			
Indian sector			
- north of STF	0.77±0.03[f]	0.55±0.03[f]	This study
- south of STF	1.00±0.07[g]	0.82±0.09[g]	

Table taken from Sherrard et al. [34]

Values are reporteds as mean±SD; or [#] average of two values

[a] From waters below 2000 m, $n = 15$

[b] From depth of 1990 m, $n = 4$

[c] From waters below 1000 m, $n = 20$

[d] From waters below 2000 m, $n = 13$

[e] From waters below 2000 m, $n = 6$

[f] From waters below 2000 m, north of the STF (42°S), $n = 15$

[g] From waters below 2000 m, south of the STF (42°S), $n = 32$

Plotting the ratio of Se(IV) to Se(VI) with depth along the transect (Figure 3.14) shows the lowest ratios are typically found in the shallow waters at the northern end of the transect. The ratio decreases as Se(IV) is selectively utilised by phytoplankton, so it would be expected that surface waters would have lower ratios. However, the values at the southern end of the transect only vary slightly over the water column, possibly a reflection of the lower productivity and low Se(IV) uptake. The Se(IV) to Se(VI) ratio in the surface waters could be dependent on primary productivity of the region and the other variables limiting productivity. The values in deep waters are

first seen to increase south of the S-STF (~42°S) (Figure 3.3), they increase southward towards parity, which has not been seen elsewhere (Table 3.2). At the southern end of the transect, the deep water ratio decreases slightly, possibly indicating recently subducted surface waters.

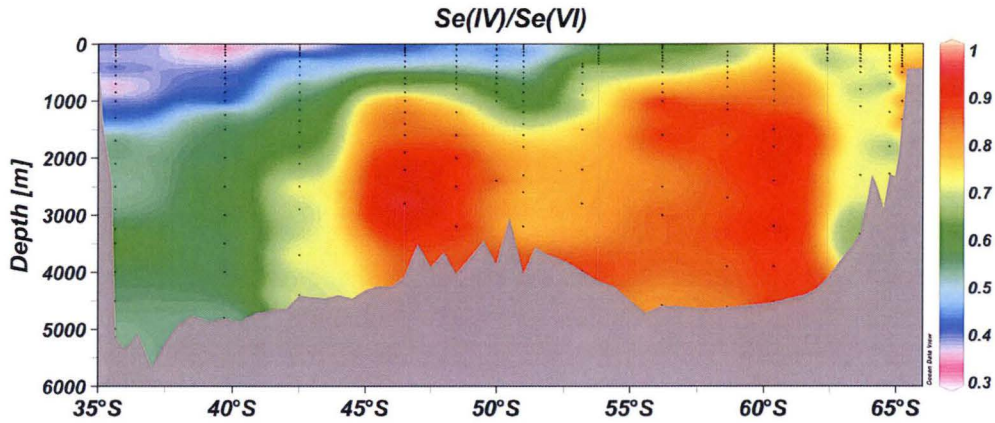

Figure 3.14 Ratio of selenium(IV) to selenium(VI) with depth for the CLIVAR I9S transect

3.3.9 Selenium and macronutrients

Previously it has been reported that Se(IV) is seen to correlate with Si, and Se(VI) with P [26]. The data for this transect is shown in Figure 3.15. When both Se(IV) and Se(VI) are correlated with P up to ~0.3 nM Se(IV) and ~0.5 nM Se(VI), there is a flattening of the curve as Se continues to increase above these levels, but P has reached its maximum concentration. Very similar curves are seen for the Se species and N. N and P were very closely linked on this transect and these curves reflect this. The flattening of the curve indicates that the remineralisation of Se is continuing longer than for these two macronutrients, suggesting Se may be more associated with skeletal rather than soft tissues components of the cell. Further investigation into where Se is used in the phytoplankton cell is required to see if this theory is valid.

There is no flattening of the top of the curve for Si and Se(IV), but both Se species are seen to increase (below ~0.2 nM for Se(IV) and ~0.4 nM for Se(VI)) before Si concentrations start to increase, resulting in a flattening at the low section of the curve. This can be explained because Si concentrations do not rise above 10 μM in the top 50 m of the water column until south of 57°S (Figure 3.15). The Se(VI) curve shows a flattening above ~0.6 nM Se(VI), these Se(VI) are from the southern end of the transect, in deeper (but not the deepest) water (Appendix 1).

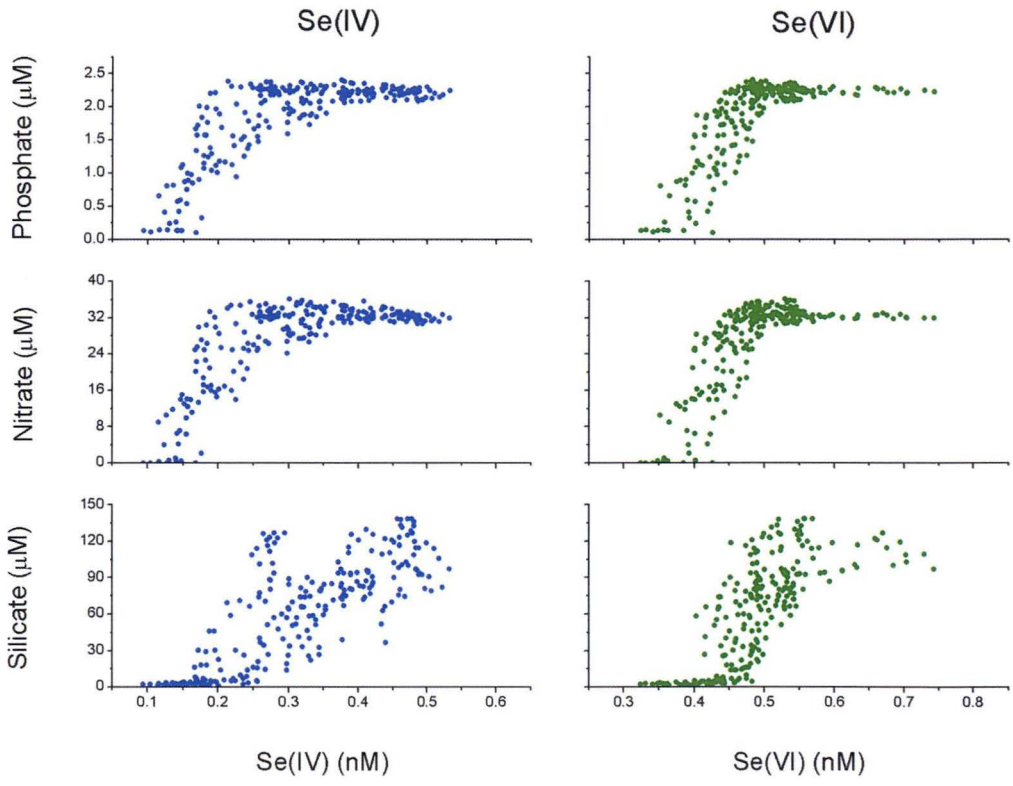

Figure 3.15 Inorganic selenium species and macronutrient correlations

Multiple linear regression (as was previously used to tease out further information on this relationship [26]) was applied to the data. The results are presented in Table 3.3. The results show that neither inorganic Se species show a strong correlation with Si or P. The previous dataset (from Measures et al. 1980 [26]) was from two cruises; whilst Measures et al. 1983 [29] was for over 400 measurements from the three

ocean basins. There is a large difference between each calculated correlation. The data from CLIVAR I9S again provides a different number; further work needs to be done to investigate whether the correlation with nutrients varies with water mass and the varying biological species present in each region.

Table 3.3 Multiple linear regression of selenium and macronutrients

Equation	r^2 (*n*)	Ref.
Se(IV) = 147.9 + 146.2P +12.1Si + 1.8N	0.67 (242)	This study
Se(IV) = -2.2 + 59.5P + 7.1Si	NR	[26]
Se(IV) = 63 + 50P + 4.2Si	0.94 (466)	[29]
Se(IV) = 121.8 + 53.2P + 1.5Si	0.66 (242)	This study
Se(IV) = 38.5 + 13.4Si	NR	[26]
Se(IV) = 187.4 + 2.1Si	0.63 (242)	This study
Se(IV) = 55.8 + 138.8P	0.52 (242)	This study
Se(VI) = 375.4 – 3.9P + 0.9Si + 2.4N	0.57 (242)	This study
Se(VI) = 352 + 3.9P + 286Si	NR	[26]
Se(VI) = 448 + 181P + 3.3Si	0.83 (409)	[29]
Se(VI) = 370.2 + 35.8P + 0.8Si	0.57 (242)	This study
Se(VI) = 547 + 301P	NR	[26]
Se(VI) = 334.0 + 82.8P	0.47 (242)	This study
Se(VI) = 414.3 + 1.2Si	0.53 (242)	This study

NB: Se are pmol l^{-1}, nutrients are μmol l^{-1}

NR – not reported

3.3.10 Organic selenium

The application of different methods, different regions surveyed, the lack of certified reference materials, and no international intercalibration of methods has resulted in uncertainty in reported Se values. The determination of Se is difficult as many methods rely on quantification by difference. There are many different methods of detection but more concerning is the different methods for species conversion (as was discussed in Chapter 2) and the lack of uniformity. An error in converting total Se or Se(VI) to Se(IV) will result in incorrect reporting of Se(VI) and organic Se. Much of the literature is best viewed for Se(IV) and total Se, due to the large discrepancies in reported values for organic Se.

Organic Se has been reported in the surface waters, decreasing with depth and not observed in deep waters (with the exception of recently subducted surface waters [28]). Its residence time has been estimated as 10.3 years in oxic waters [28]. However, there are a number of reports where organic Se increases with depth and high levels are seen in deep waters [62, 63]. This presence of increased organic Se in deep waters could be explained by suboxic or anoxic water masses [41], but with a reported consistent increase with depth, this is not possible. The lack of information on what type of species comprise the organic Se pool and the low levels at which it is present in open ocean waters hampers our ability to directly determine organic Se.

However, Aono et al. [64] determined Se-amino acid compounds, in samples from the Bering Sea, using a fractional separation method. Concentrations were low (4-20 pM), and they report that the Se-amino acid compounds account for less than 3% of the measured organic Se (measured as difference between inorganic Se species and total Se, with total Se measured by HPLC after wet ashing decomposition with concentrated nitric and perchloric acids [65]) in the same samples.

The study by Aono et al. [64] shows organic Se compounds to be at very low concentrations at their study site. Further argument against the high values of organic Se seen in some studies, is that the concentration of carbon in seawater is ~1 mg l^{-1}, and using the relative abundances of the elements, the Se/C ratio is 1×10^{6}, which equates to less than 13 pM Se l^{-1} as the organic form [26]. Further study is required

to clarify what is being measured as 'organic' Se and if it is a species of interest or an analytical artefact.

In this study, organic Se was determined by difference (subtraction of the concentration of the inorganic Se species from total Se). The highest value for organic Se was 0.09 nM. However, the method analytical uncertainty is ±0.04 nM. In the surface waters, organic Se was often seen at a similar concentration to Se(IV), the closest values were 89.6 pM organic Se to 94.6 pM Se(IV) at the northern most station (~35°S), but organic Se never exceeded Se(IV). The amount of organic Se as percentage of total Se, was highest at the northern end of the transect and in the surface waters (Figure 3.16). It has previously been reported that organic Se was 28% of total Se in Antarctic coastal waters [39]. This study did not show this higher percentage, with Antarctic coastal waters having less than 10% organic Se.

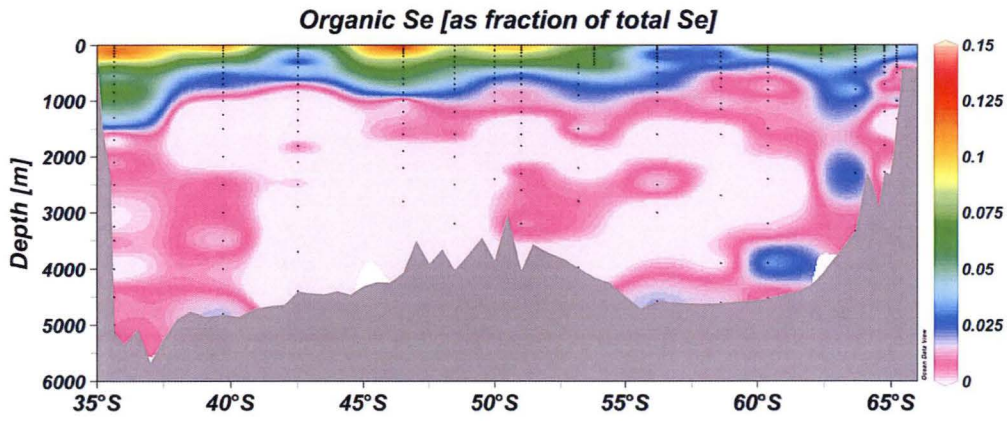

Figure 3.16 Organic selenium as a fraction of total selenium along the CLIVAR I9S transect

The data from our study does not clarify whether organic Se is a significant Se fraction or an artefact of the analytical methods used. It appears that organic Se is present in the surface waters (Figure 3.9), but is not a major contributor to total Se as has been reported elsewhere [51]. There does appear to be some link to surface waters, whether a result of productivity or an analytical artefact due to the properties

of surface waters. At the southern end of the transect, slight increases are seen in the deep water that were not seen elsewhere (Figure 3.16), possibly linked to recently subducted surface waters.

3.4 Conclusions

The study of Se on the I9S transect provides a new important and comprehensive dataset for Se. The transect allowed the collection of samples over the full water column in each of the Southern Ocean provinces. The increase of Se concentrations southwards indicate Se should not be limiting to biology. This will be further investigated in Chapters 4 and 5.

The link between the O_2 minimum zone and increasing Se(IV) concentrations was not seen as strongly here as it has been in other regions. The ratio of Se(IV) to Se(VI) in the deep waters is comparable with previous reports. Lower values were seen in the surface waters at the northern end of the transect, creating some doubt about the ratio operating, as earlier postulated, for trends with age of water masses, at least in the Southern Ocean. The link with the macronutrients did not show as strong a correlation as previously. The different water masses present, along with the different biological species composition, may have influenced this relationship.

This dataset provides a significant amount of new Se information, but new information raises many more questions about this element for which additional data is needed to permit more definitive discussion about its biogeochemical cycling in the oceans. Additional studies such as this one are required, under the GEOTRACES program (www.geotraces.org).

3.5 References

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Chapter 4

Selenium requirement for selected temperate and polar phytoplankton

4.1 Introduction

Selenium (Se) is known to be an essential micronutrient for humans, livestock and some plants. It is necessary for the formation and function of at least 13 proteins [1] in the human body. Se-containing amino-acids, proteins and enzymes have been identified, with Se-cysteine being called the 21st amino acid because of its importance in certain enzymes [2-4]. The replacement of Se-cysteine by cysteine in enzymes causes a reduction in catalytic activity compared to those containing Se [5-7]. Glutathione peroxidase, a Se-enzyme in animals, has been identified as both Se-dependent and Se-independent in plants. In plants, the Se-independent enzyme has been reported as being significantly larger than the Se-dependent type [8]. This has implications for small phytoplanktonic cells. If they can utilise Se to increase catalytic activity they will require less enzyme and maintain smaller size [6].

Se is routinely added to phytoplankton culture media. Work by Keller and colleagues in the 1980s [9, 10] developing a new medium for oceanic phytoplankton, found Se (and other microelements) to enhance growth. Se(IV) was the most significant addition to this medium. The modification of medium over the years has been made to more closely match real oceanic water concentrations, by adding more of the micronutrients, to ensure optimum growth of phytoplankton species. These micronutrient additions may now be necessary due to improved quality of reagents, minimising trace contamination from impurities in the reagents that previously provided micronutrients at sufficient levels [11].

Se addition for culturing is commonly Se(IV). Whilst this may not be representative of the chemical speciation in the environment (for details of oceanic concentrations refer to Chapter 3), Se(IV) has been shown to be the more bioavailable of the two inorganic species. Studies of phytoplankton blooms have shown greater decreases in surface-water concentrations of Se(IV) than Se(VI) [12]—presumably arising from biological uptake. Laboratory studies have confirmed that Se(IV) will be preferentially utilised even when at lower

concentrations than Se(VI) [13-15]. In fact, Vandermeulen and Foda [14] suggest that Se(VI) is actively excluded from the phytoplankton cells.

Organic Se may be an important source of Se in the surface waters of the oceans. However, whilst organic Se has been measured in these waters, individual species have not been identified. The organic Se found in the ocean is a complex mixture of many different species [16]. Baines et al. [16] utilised cell lysis to see how cell-released Se (~91% organic Se) was used by phytoplankton in comparison to Se(IV). They found the cell-released Se was taken up to roughly the same degree as Se(IV). Therefore, organic Se may also be highly bioavailable, though poorly characterised. Conversely, Cutter and Cutter [17] suggest that organic selenide is not readily taken up by phytoplankton in oligotrophic surface waters of the Atlantic Ocean. This observation was based simply on its abundance in surface waters, and no direct culture studies were performed.

4.1.1 Previous laboratory phytoplankton selenium requirement studies

Studies for the Se requirement of a wide range of phytoplankton species have been reported. Those investigating marine species are summarised in Table 4.1. As can be seen, different species within the same taxonomic class often show very different Se requirements.

For many species, growth will not occur when no or low levels of Se are added. For this reason Se is routinely added to many culture media, in the range 1–10 nM – for both artificial and natural seawater; however, media such as f/2 use natural seawater with no addition of Se, so Se occurs at natural levels. Some species demonstrate significant response to Se depletion immediately, whilst other species show delayed, subtler effects. For two species, the dinoflagellate *Katodinium rotundatum* [18] and the green alga *Dunaliella primolecta* [19], Se requirement was unclear under the conditions tested.

The biochemical role Se plays in the phytoplanktonic cell is still undefined. Some evidence suggests it is important for cell division and maintaining internal membrane integrity [20], but its exact function remains unclear. Absence of Se

can decrease exponential growth rate and cell yield. The effect of Se on cell shape can be variable. Se-depleted conditions have been seen to cause morphological changes in *Thalassiosira pseudonana* [21], where cell elongation was observed owing to inability to separate after division. Harrison et al. [18] generally observed Se-dependent diatoms to increase cell volume, primarily through increased cell length (2–5 times), in response to Se-depleted conditions, but the pennate diatom *Amphipora hyalina* decreased in cell width with a resulting decrease in cell volume. The marine dinoflagellate *Protoceratium reticulatum* also had morphological changes, including enlarged cell size, but cell shape did not appear to be distorted [22]. Conversely, another dinoflagellate *Scrippsiella trochoidea* had no visible morphological changes under Se-depleted conditions [18].

Many of the beneficial effects of Se might be explained by its role in the enzyme glutathione peroxidase [23], which is thought to contain one atom of Se per sub-unit [24]. Glutathione peroxidase, which requires Se-cysteine as part of its active site to catalyse reactions [25, 26], protects cells from damage arising from reactive oxygen species (ROS, such as peroxide and hydroxyl radicals) by reducing them [27-29]. Glutathione peroxidase activity has been identified in some phytoplankton species — marine diatom *Thalassiosira pseudonana* [30]; freshwater *Euglena gracilis* Z, *E. gracilis* var. *bacillaris* and *Astasia longa* [8, 31]; freshwater green alga *Chlamydomonas reinhardtii* [7] — but attempts to identify it in other species, for example green alga *Dunaliella primolecta* and red alga *Porphyridium cruentum* [32, 33], have failed. In these two species, Se induced destruction of peroxide by glutathione, though no comment was made on the Se requirement of these cells [32]. It has also been identified that an Se-protein is encoded in DNA, as a UGA opal codon, of *Chlamydomonas reinhardtii* [7].

Demonstrated Se requirements are difficult to compare between studies. Experimental design can affect whether a species is reported as having a Se requirement or not. For example, the diatom *Thalassiosira pseudonana* did not show any signs of limitation until a second transfer into Se-depleted media caused changes in growth rate and cell morphology [34, 35]. Work by Doblin and

colleagues [36] showed differences in requirement between different strains of *Gymnodinium catenatum*, a dinoflagellate. In the same species, Se requirement was dependent upon location and time of isolation, even when the cultures had been maintained under the same conditions in the culture collection. Therefore, comparison of reported Se requirements should be done with caution. The use of different strains, experimental variables (different media, light and temperature) and the absence of routine measurement of Se in culture media (to check the existence of variable Se background contamination as a result of the equipment and reagents used) results in great uncertainty between studies.

The published studies show that an Se requirement exists for phytoplankton – it has been found and corroborated for some species e.g. diatoms. However, it is not an exact 'science' because sometimes strains of the same phytoplankton species exhibit different requirements. In general, the majority of diatoms, dinoflagellates and prymnesiophytes have an Se requirement. There is too little information on other classes of phytoplankton to make an assessment.

Table 4.1 Reported quantitative selenium requirements of various marine phytoplankton

Species	No. of transfers	Se requirement	Ref.
Diatoms			
Amphipora hylalina	2	Yes	[18]
Cerataulina pelagica	3	No	[18]
Chaetoceros cf. *tenuissimus*	8	No	[20]
Chaetoceros debilis	2	Yes	[18]
Chaetoceros gracilis	5	No	[18]
Chaetoceros pelagica	1	Yes	[18]
Chaetoceros simplex	2	No	[18]
Chaetoceros vixvisibilis	1	Yes	[18]
Corethron criophilum	2	Yes	[18]
Coscinodiscus asteromphalus	2	Yes	[18]
Cylindrotheca closterium	5	No	[18]

Species		No. of transfers	Se requirement	Ref.
Ditylum brightwellii		1	Yes	[18]
Skeletonema costatum	NEPCC strain 18c	2	Yes	[18]
Skeletonema costatum	NEPCC strain 611	2	Yes	[18]
Skeletonema costatum	NEPCC strain 616	1	Yes	[18]
Stephanodiscus hantzschii var. *pusillus*		1	Yes	[37]
Stephanopyxis palmeriana		3	Yes	[18]
Thalassiosira aestivalis		5	Yes	[18]
Thalassiosira oceanica		2	Yes	[18]
Thalassiosira pseudonana		2	Yes	[18, 34]
Thalassiosira rotula		1	Yes	[18]
Thalassiosira weissflogii		8	No	[18]
Dinoflagellates				
Alexandrium minutum		4	Yes	[20]
Gymnodinium catenatum		2	Yes	[20]
Gymnodinium simplex		5	No	[18]
Gymnodinium sanguineum		2	No	[18]
Protoceratium reticulatum		3	Yes	[22]
Scrippsiella trochoidea		3	Yes	[18]
Prymnesiophytes				
Chrysochromulina brevefilum		?	Yes	[38]
Chrysochromulina breviturrita		2	Yes	[39]
Chrysochromulina ericina		3	No	[18]
Chrysochromulina kappa		?	Yes	[38]
Chrysochromulina polyepis		3	No	[18]
Chrysochromulina strobilus		?	Yes	[38]
Dicrateria inornata		?	Yes	[38]
Emiliania huxleyi		1	Yes	[40]
Gephyrocapsa oceanica		1	Yes	[40]
Helladosphaera sp.		1	Yes	[40]
Chlorophytes				
Chlorella sp.		1	No	[19]
Platymonas subcoriformis		1	Yes	[19]

Species	No. of transfers	Se requirement	Ref.
Cyanophyceae			
Synechococcus sp.	3	No	[18]
Raphidophyceae			
Chattonella verruculosa	2	Yes	[41]
Chrysophytes			
Aureococcus anophagefferens	4	Yes	[42]

4.1.2 This study

This study was comprised of two areas of work. The first focused on temperate phytoplankton species, and the second on Antarctic phytoplankton species. This chapter reports the results for the Se requirements for the growth of our selected species. This requirement may be seen in Se-depleted media as Liebig limitation – limitation of maximum biomass; or as Blackman limitation – limitation of the short-term growth rate.

4.1.2.1 Temperate phytoplankton

Following on from our oceanographic Se studies in the Southern Ocean (Chapter 3), where the oligotrophic waters of the subtropical zone showed the lowest Se(IV) levels in the surface water (shown further in Chapter 5), two temperate phytoplankton species – *Emiliania huxleyi* (*E. hux*) and *Synechococcus* sp. (*Syn*) – were chosen for laboratory study. The basis for this selection was that *E. hux* has worldwide distribution, excluding the polar regions [43], and *Syn* is dominant in many areas of the world oceans, except below 50°S, where the water temperature remains below 5°C year round [44]. Many Northern Hemisphere temperate oceanic species have previously been studied for Se requirements; this work investigated Southern Hemisphere oceanic strains (Table 4.2). In previous studies

Northern Hemisphere *E. hux* has been shown to have an Se requirement [40], whilst *Syn* did not show a requirement [18].

Emiliania huxleyi is a coccolithophore. Coccolithophores are characterised by an external calcium carbonate shell, which is composed of a number of plates, coccoliths (Figure 4.1a). Coccolithophores are found in the low to mid-latitudes, with *E. hux* the most widespread. Large blooms of *E. hux* are visible to satellites, as the coccoliths, which are shed from the cells, reflect light allowing the blooms to be seen (Figure 4.1b).

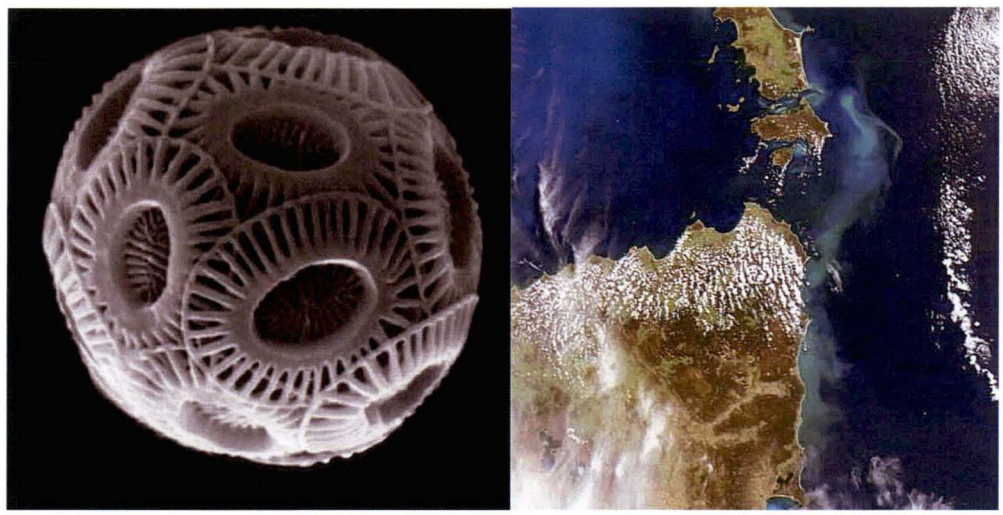

Figure 4.1a) *Emiliania huxleyi*; b) Modis visible image of an *Emiliania huxleyi* bloom, NE Tasmania, Australia (images courtesy of I. Jameson, CMAR, CSIRO)

Synechococcus sp. (*Syn*) is a cyanobacterium. It is a member of the picoplankton, which is abundant in temperate and tropical coastal and oceanic waters. *Syn* and *Prochlorococcus* represent most of the cyanobacteria in marine phytoplankton [45], and they frequently account for half or more of the biomass in oceanic areas.

The effect of Se on these cells will be measured over a minimum of three transfers via cell densities and photosynthetic parameters, an indicator of cell health.

4.1.2.2 Antarctic phytoplankton

Investigations for Se requirement were also made for four Antarctic phytoplankton species, diatoms – *Fragilariopsis cylindrus*, *Chaetoceros* sp., *Thalassiosira antarctica;* and prymnesiophyte *Phaeocystis* sp. No previous reports were found investigating polar phytoplankton for Se requirements. Many diatom species have been investigated for Se requirement previously, with the majority having such a requirement (Table 4.1). Oceanographic studies of Se have found a correlation between Se and Si – which is required for the external skeleton of diatoms (Chapter 3 and references within). Se is reported to stimulate the formation of siliceous spicules in sponges [46], leading to speculation that Se may be involved in the enzymatic synthesis of biosilica components, although in diatom exoskeletons Si deposition is a non-enzymatic process [46]. The Si requirement of diatoms and the link between Se and Si leads to the question of whether Se would be required by diatoms present in the Southern Ocean.

Diatoms are unicellular organisms, varying in size from 2 μm to chains of cells up to 1000 μm. Diatoms often dominate at temperate and high latitudes [43]; they are an important phytoplankton group, playing a key role in carbon cycling. Most diatom cells are non-motile and sink slowly. Therefore, mixing of the water column is needed to prevent them leaving the euphotic zone [47]. There are two types of diatoms, pennate and centric. Pennate diatoms often have elongated shapes. Centric diatoms are more common; they are shaped concentrically around a point.

Diatoms thrive in high-nutrient regions of the ocean, since with lower surface-area-to-volume ratios, they cannot compete with the higher bioassimilation efficiency of smaller phytoplankton in low-nutrient conditions [48, 49]. Smaller diatom species, too, typically have higher growth rates as they appear to out-compete the large-celled species.

The diatom species chosen for this study are: *Fragilariopsis cylindricus* – a sea-ice pennate diatom, which is used for paleo-reconstructions; *Chaetoceros* sp. – a centric diatom (~5 μm); and *Thalassiosira antarctica* – a centric diatom (~8 μm) (Table 4.2).

Phaeocystis sp., a prymnesiophyte (the same class as *E. hux*) is found in all oceans and is seen to bloom every summer in the Weddell Sea and Ross Sea, Antarctica [50, 51]. It forms colonies (single cells of 4–5 μm forming colonies up to 1000 μm), encased in a mucilaginous matrix, and are known for high DMS production [51]. It is also responsible for a large proportion of the CO_2 drawdown in regions of the ocean, where it is a dominant species [50].

The effect of Se on these cells will be measured over one transfer via cell densities and photosynthetic parameters, an indicator of cell health.

4.2 Methods and materials

4.2.1 Phytoplankton

All strains chosen were unicellular species (Table 4.2), except for *Phaeocystis sp.*, which was under colonial form.

Temperate species

Cultures were obtained from the Australian National Algal Culture Collection, CMAR Research Laboratories, Hobart, Australia. In the culture collection, *Emiliania huxleyi* (CS-809) is maintained in GSe medium; whilst *Synechococcus* sp. (CS-94) is maintained in f/2 medium.

Antarctic species

Fragilariopsis cylindrus and *Thalassiosira antarctica* were obtained from University of Tasmania, they were isolated by A. Pankowski from Antarctic sea ice [52]. *Chaetoceros* sp. (CS-624) and *Phaeocystis* D 4-5 (CS-243) were obtained from the Australian National Algal Culture Collection. All Antarctic strains had been growing in modified AQUIL medium (1 nM Se) for two months minimum before this experimental work.

Table 4.2 Phytoplankton strain information

Species	Origin location	Size	Source
Emiliania huxleyi	Mercury Passage, Tasmania, AUS	4-7 μm	ANACC
Synechococcus sp.	Queenscliff, Victoria, AUS	<3 μm	ANACC
Fragilariopsis cylindrus	64°S, 116°E	4-6 μm	UTAS
Thalassiosira Antarctica	64°S, 116°E	7-9 μm	UTAS
Chaetoceros sp.	Prydz Bay, Antarctica	3-5 μm	ANACC
Phaeocystis sp.	68° 47.5'S, 73° 30.2'E	4-5 μm, colony <1000 μm	ANACC

ANACC – Australian National Algal Culture Collection; UTAS – University of Tasmania

4.2.2 Culture conditions

4.2.2.1 Medium

An artificial seawater medium was used for this work. Use of an artificial medium allows better control and consistency over the course of the experiments. Modified AQUIL media was prepared [53] with modifications to the recipe summarised in the Table 4.3 (full details on the medium preparation and clean-up are in Appendix 2). The chemical speciation in the medium is shown in Table 4.3.

The major salts were made at 5-fold strength. All stock solutions for macronutrients (Chelex-extracted), trace metals, Fe-EDTA and Se(IV) (filtered at 0.2 μm) were 1000-fold concentrate and stored individually. The vitamin stock solution was that used in f/2 media (filtered at 0.2 μm); which was a 1000-fold concentrate and stored frozen.

The medium was prepared as required. The major salts were diluted to the required volume in the culture flasks and the macronutrients added, as was the buffer solution (H_3BO_3 and $NaHCO_3$). The solution was then sterilised by microwave – 4 cycles of heating on low power (90 W) – reaching a final

temperature of 96°C. Absence of bacterial contamination after the microwave treatment was verified on agar (f/2) kept at 20°C.

After cooling, the micronutrients and vitamin solutions were added. Se concentrations were varied according to the experiment and added at concentrations of 0 nM, 1 nM and 10 nM Se(IV). Media was prepared at least 5 days before use to allow equilibration between all chemical forms present.

Table 4.3 Speciation in the modified AQUIL medium and comparison with standard AQUIL and artificial seawater (at pH 7.8) (calculated with MINEQL+, version 4.5, ionic strength corrected for pH 7.8).

	Modified AQUIL		AQUIL [53]	Artificial seawater [11, 54]
Metal	Free (M)	Labile	Free (M)	Free (M)
Co	1.63×10^{-11}	2.71×10^{-11}	4.47×10^{-12}	1.20×10^{-11}
Cu	9.08×10^{-15}	2.03×10^{-14}	8.28×10^{-15}	na
Fe (Fe(III))	8.37×10^{-19}	8.45×10^{-9}	9.17×10^{-19}	1.61×10^{-17}
Se	1.00×10^{-9}	1.00×10^{-9}	1.00×10^{-8}	1.00×10^{-9}
Mn	7.49×10^{-9}	1.05×10^{-8}	7.87×10^{-9}	9.20×10^{-7}
Ni	1.14×10^{-13}	1.34×10^{-13}	na	1.55×10^{-13}
Mo	1.00×10^{-8}	1.00×10^{-8}	1.00×10^{-7}	6.10×10^{-9}
Zn	1.43×10^{-10}	2.93×10^{-10}	1.04×10^{-11}	7.81×10^{-10}
Ionic strength	*0.664*		*0.664*	*0.561*

na = not added;
Note: All Se is under its free form in these media

4.2.2.2 Growth conditions

The temperate strain cultures were grown in a 12-h light-dark cycle, at 20°C with light intensity 170 µmole photons m^{-2} s^{-1}. The Antarctic strains were grown at 4°C under continuous light (100 µmole photons m^{-2} s^{-1}).

E. hux and *Syn* were grown for at least 3 transfers; the 0 nM Se experiment was commenced at the first transfer of the replete experiments from the 1 nM Se culture. Antarctic strains were grown for one transfer only. According to growth curves, *E. hux* was transferred each 6 days and *Syn* each 5 days, to ensure cells were in exponential growth phase. Each experiment was performed at least in duplicate.

For culture transfers, the required volume of the culture was placed in a tube with 1 mM EDTA and 0.6 M NaCl solution for 5 minutes. This treatment was to remove cationic metals (except Fe) sorbed to the cell surface [55]. The solution was then gently filtered (2 µm (or 0.2 µm for *Syn*)) through a membrane filter on a Sartorius filter unit with vacuum hand pump (15 mm Hg vacuum maximum), and rinsed with chelex-extracted 2.38 mM $NaHCO_3$ and 0.6 M NaCl solution (2-3 ml twice). The filter was placed in a tube and the phytoplankton gently resuspended with the required volume of the $NaHCO_3$ and NaCl solution. This solution was then transferred to the new experimental flasks. Minimal additions were made to prevent dilution of the medium, typically a 1-in-20 addition.

Cultures were grown in 60-ml polystyrene flasks or 125-ml polycarbonate Erlenmeyer. Before use, flasks were 10% v/v HCl-washed for 1 week and Milli-Q rinsed (7 times).

Cultures were not axenic, but care was taken to minimize bacterial contamination. All manipulations were done under a HEPA laminar flow-hood. All filters, tubes and tips used were washed in 10% v/v HCl.

4.2.3 Analyses

4.2.3.1 Growth rate

All cultures were sampled routinely for cell counts (particle counter – Coulter Counter Multisizer II, Beckmann, 50-µm aperture tube). Culture samples (0.2–1.0 ml depending on cell density) were diluted with 9 ml of electrolyte solution (3% m/v NaCl and 0.1% m/v NaN_3). Cell size was also measured by the particle counter.

Phaeocystis sp. was treated slightly differently, as the colonies caused inconsistent readings with the particle counter. Thirty microlitres of glutaric dialdehyde (50% w/w solution, Aldrich Chemical Company) was added to 1 ml of sample and vigorously shaken so as to break the colonies. The sample was stored at 4°C for at least 24 h. It was then shaken, diluted with 9 ml electrolyte solution, and counted.

Growth rate was calculated from changes in cell density, using the formula:

$$\mu\,(\text{day}^{-1}) = \frac{\ln N_1 - \ln N_2}{\Delta \text{days}} \qquad \text{(Eq. 1)}$$

μ - growth rate, N – cell density

4.2.3.2 Photosynthetic parameters

Photosynthesis is a complex chemical reaction that requires light absorption, water and carbon dioxide to produce sugars and other organic molecules. Micro-organisms, such as autotrophic phytoplankton rely on photosynthesis as their carbon source.

$$6H_2O + 6CO_2 \xrightarrow{h\nu} C_6H_{12}O_6 + 6O_2$$

Photosynthesis occurs in the thylakoid membrane, which is in the chloroplast (Figure 4.2). It requires two assembled proteins: photosystem I (PSI) and

photosystem II (PSII). PSII consists of a photoreceptor antenna, such as chlorophyll *a* (chl *a*), which captures photos at 680 nm. PSI is formed from chl *a* associated with various proteins and absorbs light at 700 nm.

During photosynthesis, photons are captured in the antenna of the photoreceptor of PSII; this is the energy absorption step. Some of this energy can be lost as heat and/or fluorescence (Figure 4.3). Energy not lost will excite an electron that is transferred to a reaction centre composed of two chl *a* molecules (P_{680}); this is the electron or energy capture step. The plastoquinone (PQ) captures the electron from the P_{680} and transfers them to PSI; the electron or energy transport step. The PSII is compensated for the electron loss by water hydrolysis to H^+ and O_2. The electron transport from PSII to PSI generates a proton gradient through the thylakoid membrane, activating the ATPsynthase generating ATP in the chloroplast.

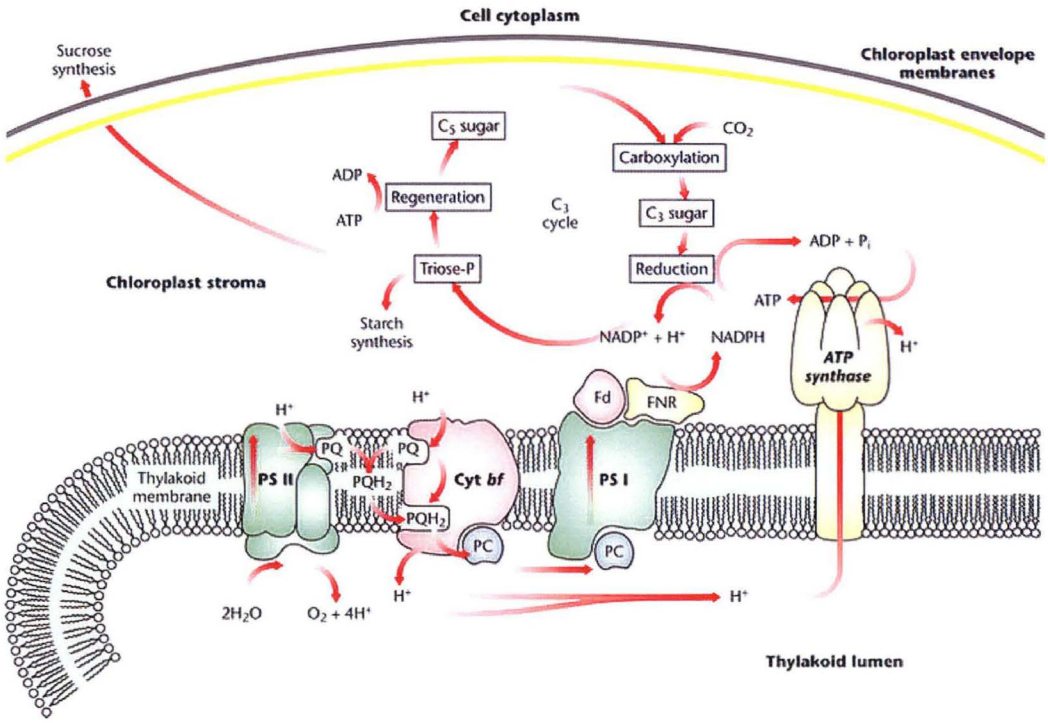

Figure 4.2 Representation of the photosynthesis reaction, including the Calvin Cycle (C3 cycle) (image courtesy of S. Grace, University of Arkansas)

Photons can also be directly captured by the antenna of PSI. In this case, the electron will cause the formation of NADPH. Both ATP and NADPH are used in the second phase of the photosynthesis for the transformation of CO_2 into more complex sugars, which is known as the Calvin Cycle [56] (Figure 4.2).

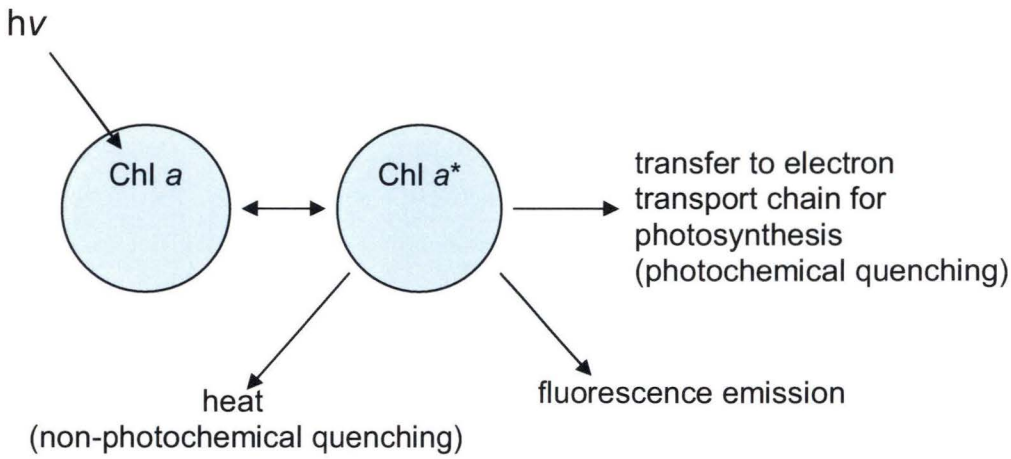

Figure 4.3 Possible fate of electrons once captured by chl *a*. The excited chl *a** will emit fluorescence and can either transfer its electron to the electron transport chain to drive photosynthesis; or lose its electron via heat production.

The activity of PSII was studied using a PhytoPAM and PhytoWin software (version 1.46). Three millilitres of sample was collected in a quartz cuvette (acid cleaned) and placed in the PhytoPAM detector. This system uses three different light wavelengths to distinguish brown, green and blue algae. It measures the fluorescence emitted before (F_0) and during (F_m) short high light excitation (10000 µmole photons m^{-2} s^{-1} light pulse for 0.6 seconds). When the light is absorbed, chl *a* becomes excited. The electrons are then transferred to the PQ, stabilizing the system. When all PQ is reduced, there are no available electron receptors in PSII and all reaction centres are closed. Additional electrons return to their stable unexcited state with the emission of a photon, providing either heat or fluorescence [57, 58].

Measurement of rapid light curves and quantum yield were made to assess the photosynthetic health of the cells.

4.2.3.2a Quantum yield measurement

Cells were dark adapted (60 min), to ensure no electrons remained in the transport chain and all chl *a* were unexcited. A weak measuring light (0.15 µmole photons m^{-2} s^{-1}) was applied to induce fluorescence emission (F_0) without causing any photosynthetic reaction. A light pulse was applied causing all reaction centres to capture electrons and remain closed until they transfer along the electron transport chain. The fluorescence was measured (F_m). The maximum quantum yield is given by:

$$F_v/F_m = (F_m - F_0)/F_m \qquad \text{(Eq. 2)}$$

F_v/F_m is related to the number of reaction centres participating in the light capture. Typically a maximum value of 0.65 is measured, with the PhytoPAM, attesting to optimal photosynthetic efficiency.

4.2.3.2b Rapid light curves

The PhytoPAM possesses an additional light source (actinic light), which allows the study of the photosynthetic apparatus under variable light levels (0-2000 µmole photons m^{-2} s^{-1}). Rapid light curves (RLC) present the measurement of basal (F') and maximal (F'_m) fluorescence levels at increasing light intensities. Light intensities are increased, step-wise, at 30-s intervals, each ending with a saturating light pulse. The parameters derived from RLC do not represent a steady state of PSII as in conventional photosynthetic irradiance curves, but a short term response to light stress [58].

Usually quantum yield will decrease and relative electron transport rate (rETR) will increase with increasing light intensities until a plateau is reached. Three different regions are depicted in a RLC (Figure 4.4):

a) light-limited where rETR increases linearly with photosynthetic active radiation (PAR)
b) light-saturation where rETR reach a maximal plateau
c) photo-inhibition, where rETR is diminished due to excess light.

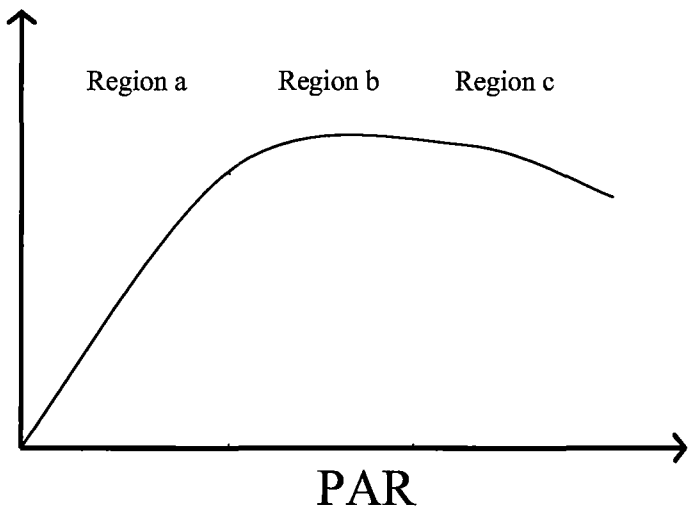

Figure 4.4 Example of a rapid light curve indicating the different regions as described in the text

Relative electron transport rate is calculated as follows:

$$rETR = [(F'_m - F')/F'_m] \times PAR \qquad \text{(Eq. 3)}$$

$(F'_m - F')/F'_m$ is the effective quantum yield, indicating the amount of energy used in photochemistry.

Three main parameters are calculated from the RLC using the automatic non-linear fitting function of PhytoWin:

$rETR_{max}$ – the maxiumum level of rETR (derived from region b of the RLC). $rETR_{max}$ is the maximum rate of electrons being transported through the photosynthetic chain.

α – slope of region a, it is proportional to the efficiency of light captures (and thus effective quantum yield).

E_k – minimum saturating light defined as $rETR/\alpha$. E_k is related to the quenching induced during the RLC, where photochemical quenching dominates below E_k and non-photochemical quenching dominates above it. Above E_k the reaction centres are saturated so most of the energy from the excited chl *a* will be lost as heat.

4.2.3.2c Chlorophyll a

Samples for chl *a* were collected by filtration of 5 ml of sample on 25-mm GF/C filter. The filters were stored at –80°C until analysis. Chl *a* was extracted in low light with 9 ml of 90% acetone. Samples were sonicated for 15 min in ice, and placed in the fridge for 20 h. Samples were then allowed to warm to room temperature (30 min in the dark), before being centrifuged (2500 rpm for 5 min). Samples were analysed by Fluorometer AU-10 (Turner Designs) with emission and excitation filters ($\lambda_{excitation}$ 436nm, $\lambda_{emission}$ 680 nm) for non acidified chl *a* determination [59]. The fluorometer was calibrated using certified chl *a* standard solutions (Aqualab Scientific).

4.2.3.2d Pigments

Pigment analysis was carried out for the Antarctic strains. 5 ml of sample was filtered through a 25-mm GF/C filter and stored at –80°C until analysis. Samples were extracted over 15–18 h in an acetone solution (100%, diluted to 90% for analysis, Mallinkrodt, HPLC grade) before analysis by HPLC using a C_8 column and binary gradient system with an elevated column temperature (55°C) following a modified version of the Van Heukelem and Thomas (2001) method [60]. Pigments were identified by retention time and absorption spectrum from a photo-diode array (PDA) detector and concentrations of pigments were determined from commercial and international standards (Sigma; DHI, Denmark).

4.2.3.3 Selenium – dissolved

Dissolved Se was measured by HG-CT-AFS, as described in Chapter 2. Samples were filtered on 0.2 μm polycarbonate filters (acid-cleaned; Sartorius).

4.2.3.4 Viscosity measurements

Viscosity measurements were made on some *Syn* samples to check if exopolysaccharides were being produced and causing the observed aggregation of cells. Culture subsamples were centrifuged for 10 min at 2000 rpm, 450 x g at either 2 or 20°C (Sorvall RT6000, DuPont, Wilmington DE, USA). A 1 ml sample was placed in the sample cup of a Brookfield microviscometer (Model LVT, Brookfield Engineering Laboratories, Inc., Stoughton MA, USA) connected to a recycling water bath (BCT-9090, Thermoline, Smithfield, NSW, Australia), which maintained the temperature of the samples at 25°C during viscosity measurements. Speed was set at 30 rpm. Sample temperature was allowed to equilibrate for 2 min and the viscosity was read three times, once after each of 3 sets of 10 revolutions. The cup and cone were cleaned with ethanol/water solution (70/30; v/v) between samples.

4.3 Results and discussion

4.3.1 Temperate strains

4.3.1.1 Viscosity measurements

Syn were seen to be forming aggregates after 6 days (Figure 4.5). The reason for this was unknown. This species is routinely grown in f/2 medium (CSIRO Marine Algal Collection), which is a richer medium than the modified AQUIL being used in these experiments. There are reports of some S*ynechococcus* strains suffering from photo-inhibition [61, 62]. To ensure this was not occurring, or causing the aggregation, a lower light level (80 µmole photons m^{-2} s^{-1}) was tested. The f/2 and modified AQUIL media were tested at the two different light levels to see if a reason for the aggregations could be determined. Viscosity was measured to verify whether aggregation was a result of excretion of substance such as polysaccharides, that could increase viscosity in the medium [63]. The samples were tested and compared to stock culture (f/2 medium) for viscosity with no significant difference being seen between any of the different *Syn* culture conditions (Table 4.4)

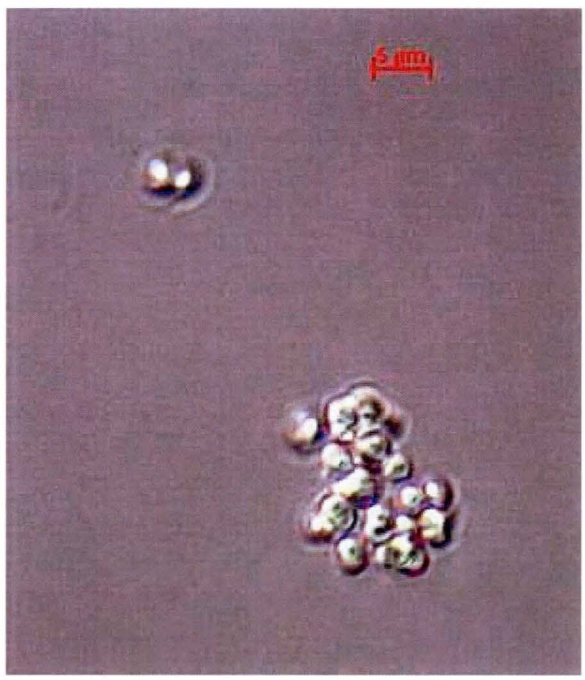

Figure 4.5 Aggregation of *Synechococcus* sp. cells

Table 4.4 Viscosity measurements of *Synechococcus* sp. cultures in different media and with different light treatments

Sample	Light	Media	Viscosity (cpS)	
			20°C	2°C
Control		f/2	0.9	
Milli-Q			0.9	
CS-94		f/2	1.1	1.0
	Low	f/2	0.9	1.1
	High	f/2	1.1	1.3
1 nM Se	Low	Modified AQUIL	0.9	0.9
1 nM Se	High	Modified AQUIL	1.0	1.0
1 nM Se	High	Modified AQUIL	0.9	0.9
10 nM Se	High	Modified AQUIL	0.9	1.0

High light = 170 μmole photons m^{-2} s^{-1}; low light = 80 μmole photons m^{-2} s^{-1}

No cause for the aggregation could be seen due to increased viscosity in the medium. The cells did not show signs of photo-inhibition at the higher light level in modified AQUIL medium (μ = 0.50–0.51 d^{-1} for both light treatments). *Syn* were routinely transferred at 5 days, to ensure the cells were still in exponential growth and that aggregation had not started to occur.

4.3.1.2 Media

The culture medium was carefully prepared to minimise any possible bacterial or trace metal contamination. The medium was tested for Se contamination. Various batches were tested in order to ensure consistency across growth experiments. Se was below the detection limit of the HG-CT-AFS system (<5 pM).

4.3.1.3 Growth rate and cell volume

The growth rate and maximum biomass of *E. hux* was reduced when no Se was added to the culture media (Figure 4.6 & Table 4.5). No difference was seen between additions of 1 nM and 10 nM Se, indicating that the Se growth requirement is met by addition of 1 nM Se. Growth rate, in 1 nM and 10 nM Se addition media were constant over time, whilst the growth of cells in 0 nM Se addition was reduced and continued to decrease. In the previous study of *E. hux* Se requirement [40], the strain tested did not show a difference in growth rate (between 0 nM and 10 nM Se(IV)), rather growth ceased at 90 h (3.75 d) in Se-depleted medium.

Table 4.5 *Emiliania huxleyi* growth rates

Treatment		μ d^{-1}
0 nM Se	1st growth cycle	0.31±0.09
	2nd growth cycle	0.31±0.03
	3rd growth cycle	0.19±0.04
1 nM	All	0.74±0.05
10 nM	All	0.75±0.05
Addback	1st growth cycle	0.88±0.01
	2nd growth cycle	0.60±0.22

Cell volume varied most significantly in cells grown in Se-depleted medium (Figure 4.6). This change was most noticeable in the first growth cycle in Se-depleted medium. It was present in subsequent growth cycles but cells did not reach the maximum volume seen in the first growth cycle. This might be due to the decrease in growth and cell numbers in successive growth cycles.

Microscopic observations did not show any variation in the number of coccoliths (as layers on the cells) between the Se-replete conditions. However, visually there was a difference between the treatments (Figure 4.7). This may be due to an increase in the number of shed coccoliths. The Se-depleted condition had decreased number of coccoliths but this was thought be due to lower cell density. The amount of calcification was not standardised to cell numbers. Work by Fabry [64] indicates that for a high calcifying *E. hux* strain, coccolith formation was at least three times higher when grown in medium (K-media) with Se (1–2 nM Se(IV)) than in Se-depleted media. The shape and size of the coccoliths was not studied, so it is unknown if changes occurred here.

Small fluctuations in cell volume were seen for Se-replete cells. Cell volume was seen to increase in the early stage of the growth curve and then decrease during exponential cell growth; this is associated with normal growth. As can be seen in Figure 4.6, the fluctuations in cell volume were very similar for the two Se-replete conditions. The decrease in growth rate and the increase in cell volume suggest that the cells are unable to divide properly when Se-depleted (especially for the first growth cycle in these conditions). This has been previously observed for diatom *Thalassiosira pseudonana* [21] and dinoflagellate *Protoceratium reticulatum* [22], but not reported for *E hux*. This is in contrast to the reported effect on cells of other micronutrient limitation. For example, iron limitation was shown to diminish diatom cell size [65]. Why this increase in cell size is seen to decrease on transfer is unknown – the use of an EDTA wash during the transfer may have destabilised the cell, or transfer to new medium may stimulate initial growth by the small background contamination of Se in the medium.

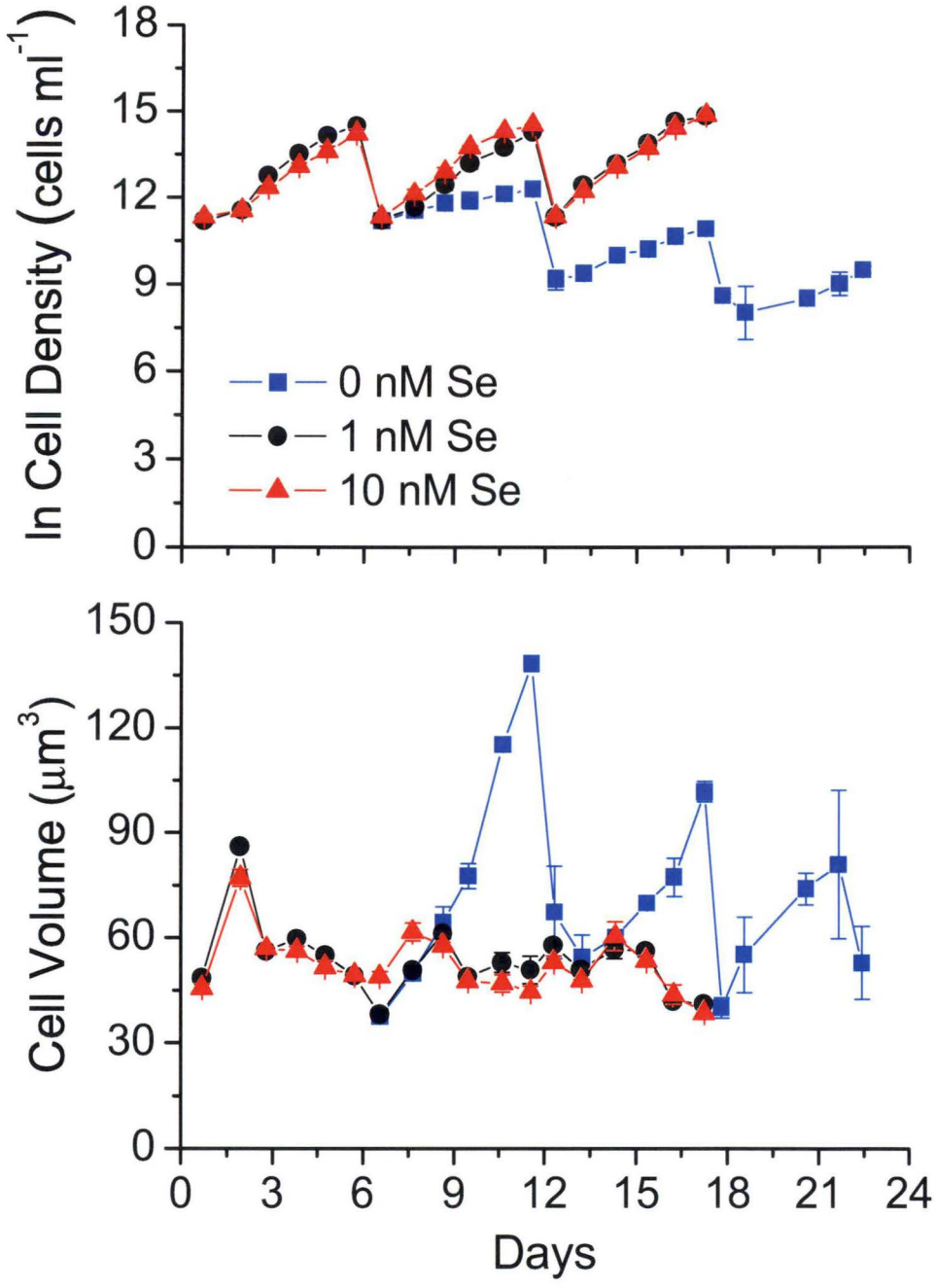

Figure 4.6 Effect of varying selenium concentration on growth and cell volume of *Emiliania huxleyi* ($n = 3$). NB: 0 nM Se is plotted from day 6 as the experiment was commenced at this time.

Figure 4.7 Visual difference between *Emiliania huxleyi* cultures in different selenium concentrations

The growth of *Syn* did not appear to be affected by different Se concentrations (Figure 4.8). Growth rates were constant among different Se treatments and over time (μ = 0.51–0.57 d^{-1}), as previously observed for this cyanobacteria [18]. Small fluctuations in cell volume were seen for all Se treatments (Figure 4.8). These were ascribed to normal fluctuations in growth and were similar across all Se concentrations.

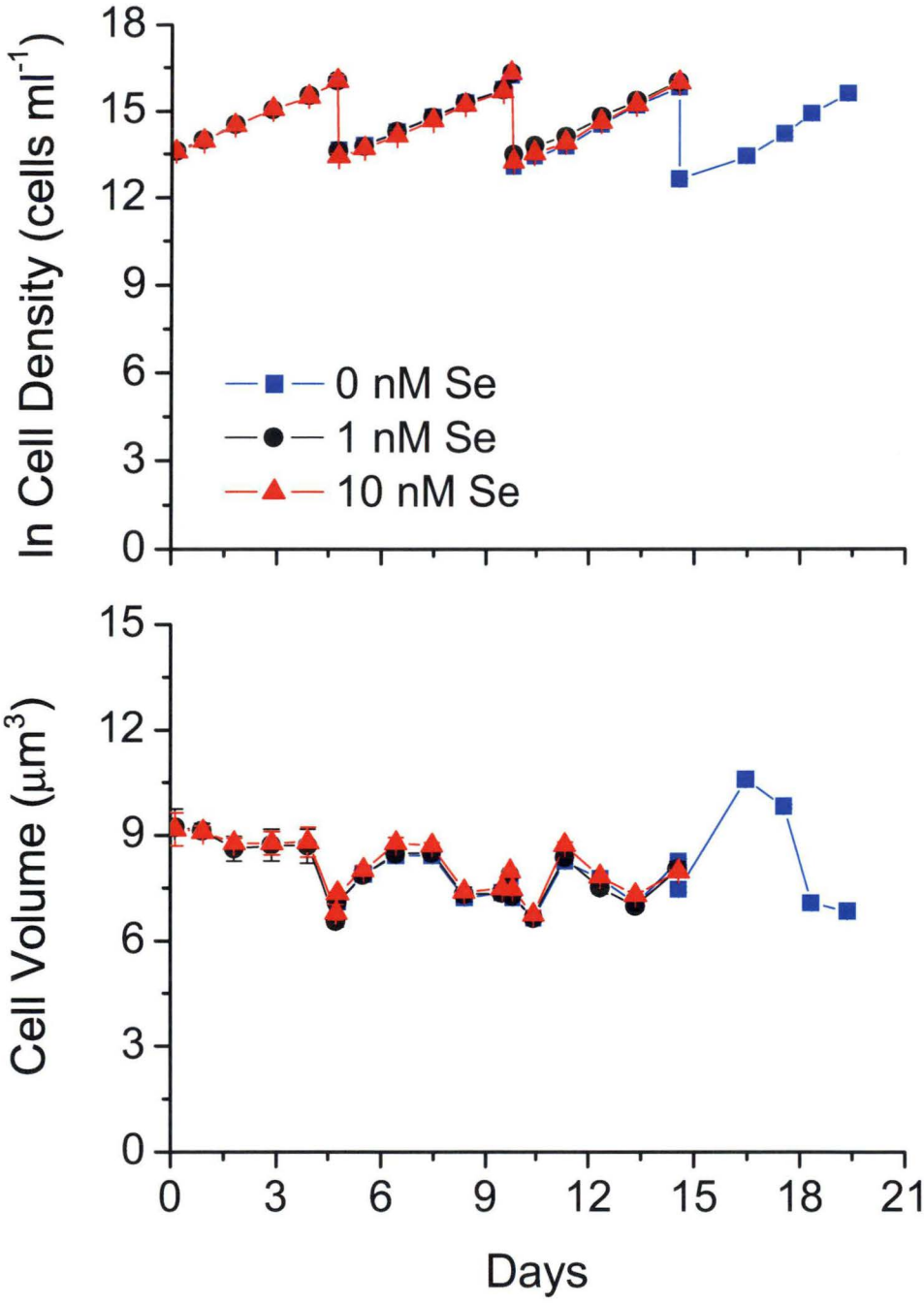

Figure 4.8 Effect of varying selenium concentration on growth and cell volume of *Synechococcus* sp. (n = 3). NB: 0 nM Se is plotted from day 6 as the experiment was commenced at this time.

4.3.1.4 Add-back experiments

As Se depletion in natural systems may be transient, the *E. hux* requirement for Se was investigated in additional experiments to see if the cells could recover after being Se-depleted. Danbara and Shiraiwa [40] reported that *E. hux* could recover from Se-depleted conditions (~7 d) when transferred to Se-replete medium (10 nM Se addition). Diatom *Thalassiosira pseudonana* was able to recover from Se-depletion (two growth cycles, with Se-deficiency not evident until the second growth cycle) when transferred to 1 nM Se media, but was often unable to recover if Se-depleted for three growth cycles [34, 35]. Of fifteen diatoms found to have a Se requirement, Harrison et al. [18] found twelve were able to recover from Se deficiency when transferred to 10-nM Se media.

Cells that had been grown in Se-depleted media for one and two growth cycles were transferred to 1 nM Se modified AQUIL. Cells were seen to recover. Those that had been Se-depleted for two growth cycles did so to a lesser degree than those depleted for one growth cycle (Figure 4.9). This was not tested on cells that were Se-depleted for three growth cycles as cell numbers were too low. The growth rates of cells Se-depleted for one growth cycle were above those seen when cells were continually grown in Se-replete medium. The growth rates of those that had been depleted for two growth cycles showed greater variability and on average were below that seen for Se-replete cells (Table 4.5). The recovery of cells suggests that the deleterious effects of Se-depletion are partially reversible. However, following prolonged Se deficiency *E. hux* will not grow and might be unable to develop following Se enrichment.

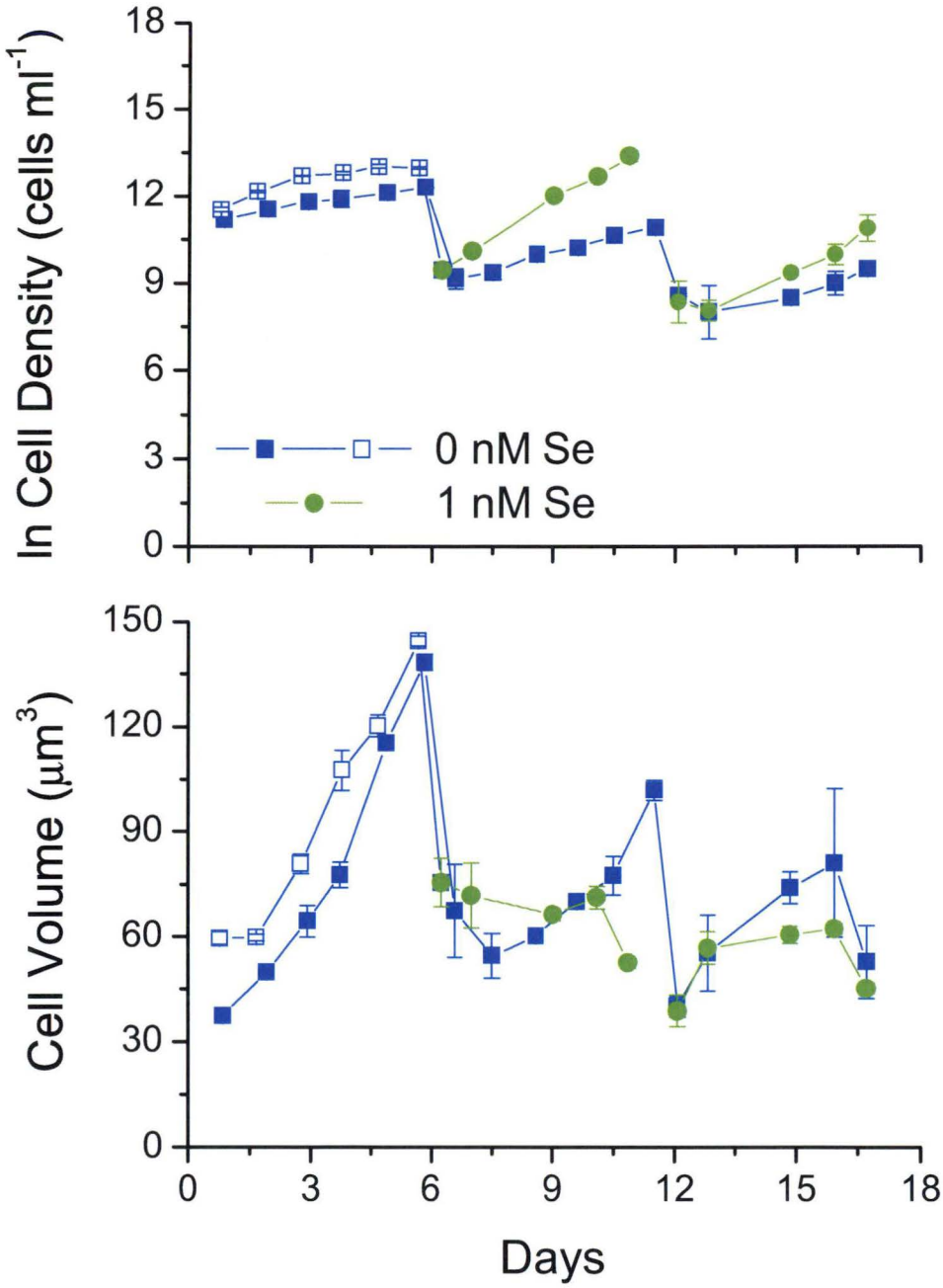

Figure 4.9 Recovery of *Emiliania huxleyi* cells with transfer from Se-depleted media to Se-replete media (n = 3). NB: Two sets of 0 nM Se data are presented as the transfers came from two different 0 nM cultures.

4.3.1.5 Photosynthetic parameters

4.3.1.5a Rapid light curve and maximum quantum yield

The health of cells was investigated by measuring the cells' photosynthetic efficiency. Higher maximum quantum yield (to a maximum value of 0.65) indicates higher photosynthetic efficiency with more reaction centres participating in light capture – equating to healthier cells. The values for *E. hux* from the RLC (Table 4.6) will be considered in two sections: first, the cells grown for three growth cycles under the same conditions (G3 values), and then the add-back experiments (AB).

For the G3 experiments, the results show that α (the efficiency of light capture under non-steady state) is not significantly different for cells grown in 0 nM and 1 nM Se. The α value for cells grown in 10 nM Se is slightly different to 0 nM Se, indicating less efficient light capture in these cells. The values for $rETR_{max}$ (the maximum rate of electron transport through the system) show that 10-nM Se cells have high transport efficiency compared to those Se-depleted cells; again cells from 0 nM and 1 nM do not show a difference. E_k values show a similar trend to those of $rETR_{max}$, with higher values for 10-nM Se cells – indicating higher light levels are required to saturate PSII of these cells. The quantum yields do not vary significantly between any of the treatments.

Table 4.6 Rapid light curve parameters and quantum yield measurements for *Emiliania huxleyi* as a function of the concentration of selenium

	0 nM Se G1 *(n=3)*	0 nM Se G3 *(n=3)*	1 nM Se *(n=2)*	10 nM Se G3 *(n=3)*	AB G1 *(n=3)*	AB G2 *(n=3)*
α	0.17 ± 0.01	0.19 ± 0.05	0.12 ± 0.03	0.12 ± 0.01	0.15 ± 0.01	0.16 ± 0.00
$rETR_{max}$	94.1 ± 17.7	62.4 ± 14.5	53.4 ± 4.0	117.8 ± 21.3	222.0 ± 35.3	136.0 ± 2.4
E_k	566.4 ± 100.5	354.3 ± 147.5	453.1 ± 84.0	981.2 ± 73.2	1432.3 ± 175.2	859.7 ± 8.2
F_v/F_m	0.53 ± 0.01	0.50 ± 0.02	0.40 ± 0.10	0.39 ± 0.09	0.55 ± 0.01	0.55 ± 0.01

F_v/F_m – maximum quantum yield following dark adaptation; $rETR_{max}$ – relative maximum electron transport rate; α is proportional to efficiency of light capture; E_k – the minimum saturating irradiance; G# – growth cycle #; AB – addback experiments, G1 = one growth cycle in Se-depleted medium; G2 = two growth cycles in Se-depleted medium

The add-back experiments showed the cells to have increased growth rate compared to those cells grown continuously in Se-replete media (Table 4.5). This increase is also seen in the RLC results, with an increase in quantum yield, $rETR_{max}$ and E_k compared to 1 nM Se G3 cells and an increase in $rETR_{max}$ and E_k compared to 0 nM Se (G1 and G3). These cells show that Se, in this case, improves photosynthesis with regard to electron transfer and maximum saturation irradiance, whilst light capture efficiency (α) is unchanged (Figure 4.10).

The previous study on *E. hux* [40] reports O_2 evolution as a measure of gross photosynthesis, which is related to ETR [58], was increased in the Se-depleted culture (0 nM Se). On transfer to Se-replete medium (10 nM Se), the values returned to those seen for Se-replete cells. This observation was confirmed by this study, with a decrease in rETR in Se-depleted media and return was seen upon transfer to Se-replete conditions.

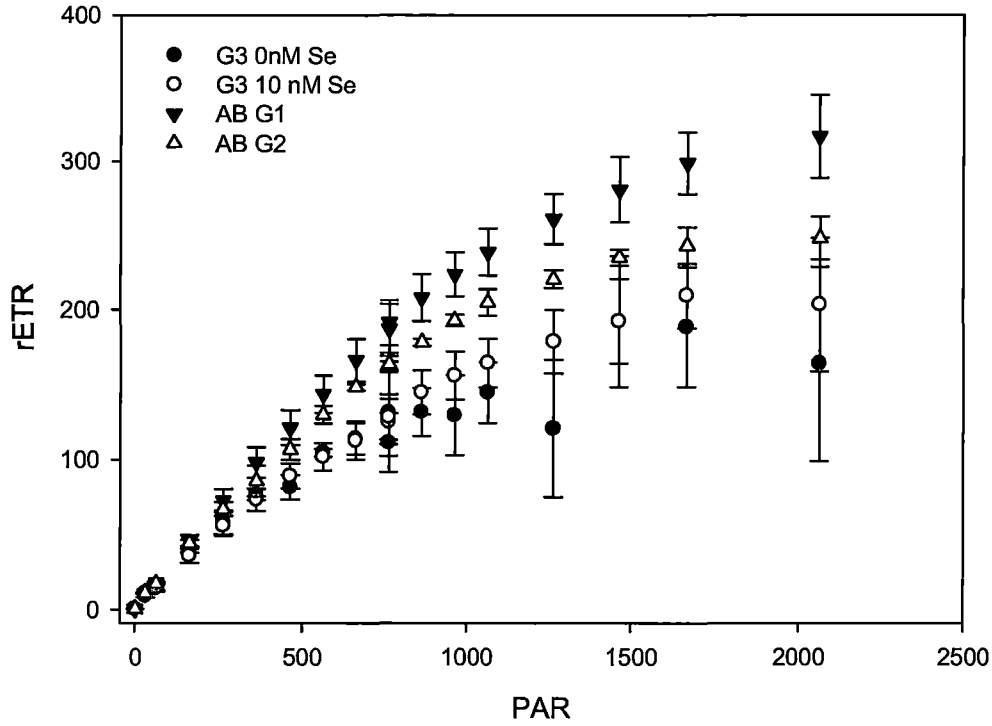

Figure 4.10 Effect of varying selenium concentration on rapid light curve of *Emiliania huxleyi*. G3 – 3 growth cycles; AB – addback experiments, G1 – one growth cycle in Se-depleted medium, G2 – two growth cycles in Se-depleted medium

The effect of Se on photosynthesis (as seen in the add-back results) was absent for cells grown continuously under 0 nM and 1 nM Se. For these cells, the effect of Se was seen as a difference in growth rate. From these results, photosynthesis is not the first site that Se acts on, as 10-nM Se was required to see a change in photosynthetic parameters.

Syn did not show any significant differences in any of the photosynthetic parameters measured (α, $rETR_{max}$, E_k and quantum yield) between different Se treatments (Table 4.7). It appears that Se did not affect the efficiency of PSII or electron transfer for *Syn*. The quantum yield was much lower than the value reported for healthy cells (~0.30 compared to 0.65), although this value was

reported for eukaryotic plankton, for example *Thalassiosira pseudonana* [66]. *Syn* grown in replete conditions and low light (10 μmole photons m^{-2} s^{-1}) were reported to have a maximum quantum yield of 0.45 [67]. These cells were grown under high light, which has been reported to reduce maximum quantum yield in *Syn* sp. [68]. Temperature has also been shown to affect quantum yield for cyanobacteria, a *Trichodesmium* strain had a quantum yield of 0.1 at 17°C compared to 0.6 at 30°C [69], and *Plectonema boryanum* UTEX 485 showed lower quantum yield when grown at moderate temperature and high light intensity [70]. No reports were found on temperature and *Syn* so it is not known if the same applies in this case.

Table 4.7 Rapid light curve parameters and quantum yield measurement for *Synechococcus* sp. as a function of the concentration of selenium

	0 nM Se *(n=4)*	1 nM Se *(n=3)*	10 nM Se *(n=3)*
α	0.16 ± 0.00	0.15 ± 0.00	0.15 ± 0.01
$rETR_{max}$	115.9 ± 16.3	106.3 ± 32.3	98.3 ± 5.0
E_k	726.3 ± 108.7	668.3 ± 168.0	657.1 ± 46.1
F_v/F_m	0.29 ± 0.01	0.28 ± 0.01	0.28 ± 0.01

F_v/F_m – maximum quantum yield following dark adaptation, $rETR_{max}$ – relative maximum electron transport rate; α is proportional to efficiency of light capture; E_k – the minimum saturating irradiance

4.3.1.5b Chlorophyll a

Chl *a* was measured and the amount per cell was calculated. For *E. hux*, Se-depleted cells had low chl *a* per cell, with Se-replete cells having higher values (Figure 4.11). The concentration of chl *a* per cell was highest in add-back experiment cells that had been Se-depleted for 1 growth cycle, with those that had been depleted for 2 growth cycles showing chl *a* levels similar to those that had been growing continuously in 1 nM Se medium.

If electron capture efficiency is related to chl *a* concentration in *E. hux*, it would be expected that higher α would be seen at higher Se concentration. This was not

seen, suggesting that *E. hux* relies on additional pigments to serve as antennas and capture electrons. However, the level of chl *a* in the cell give good correlation with $rETR_{max}$ at the same Se treatments.

Syn did not show any significant difference in the level of chl *a* per cell in the different Se treatments (Figure 4.12).

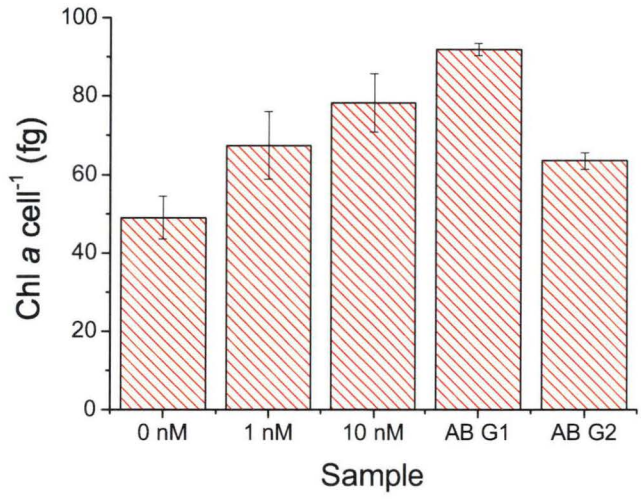

Figure 4.11 Chlorophyll *a* concentration per cell of *Emiliania huxleyi* after three growth cycles

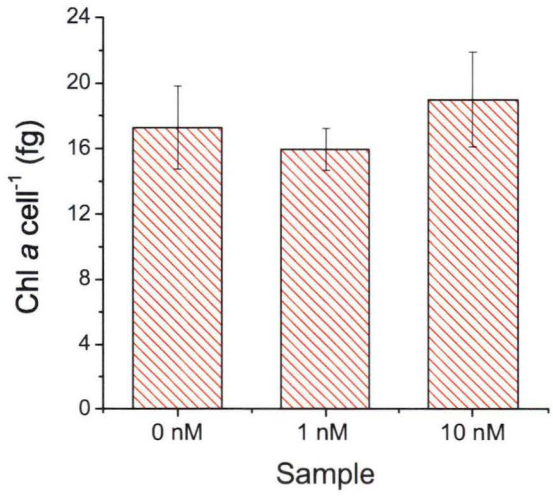

Figure 4.12 Chlorophyll *a* concentration per cell of *Synechococcus* sp. after four growth cycles, NB: different scale to that on Figure 4.11

4.3.2 Antarctic strains

4.3.2.1 Growth rate

The Antarctic strains were grown in modified AQUIL with no or 1 nM Se addition. The growth of these strains for only one growth cycle will only identify strains that have a strong Se requirement and insufficient stored intracellular Se to meet this requirement. Results are shown in Figure 4.13. *Thalassiosira antarctica* ($\mu = 0.30 \pm 0.01$) and *Phaeocystis* sp. ($\mu = 0.21 \pm 0.02$) did not show a growth requirement under these conditions. *Chaetoceros* sp. showed a large difference in growth rates ($\mu = 0.42 \pm 0.02$ and 0.21 ± 0.02, in the presence and absence of Se), indicating a strong Se growth requirement, and *Fragilariopsis cylindrus* had a smaller but still noticeable difference in growth rates ($\mu = 0.39 \pm 0.00$ and 0.29 ± 0.00 in the presence and absence of Se), showing it also to have a Se growth requirement. Both *Chaetoceros* and *Fragilariopsis cylindrus* showed limitation in the maximum biomass when grown in Se-depleted conditions (a decrease from ~1.7×10^6 cells ml^{-1} to ~0.3×10^6 cells ml^{-1} and from 2.5×10^6 cells ml^{-1} to 1.3×10^6 cells ml^{-1} in absence of Se for *Chaetoceros* sp. and *Fragilariopsis cylindrus*, respectively). *Thalassiosira antarctica* is the largest cell of the strains tested. Its growth rate is slower, meaning less Se will be utilised due to a low division rate; it may have a Se growth requirement that was not revealed under these conditions.

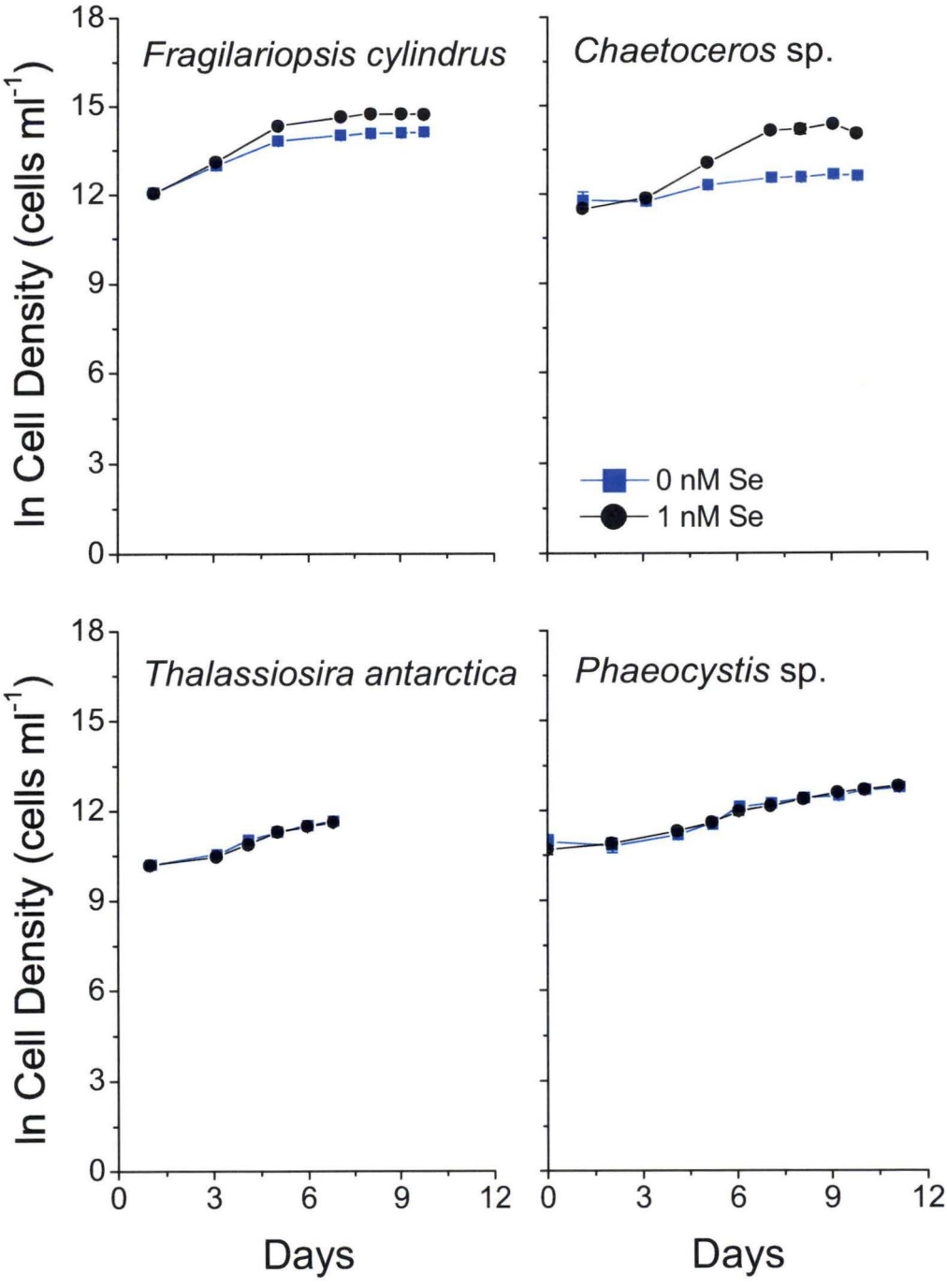

Figure 4.13. Growth of Antarctic strains with varying selenium concentrations ($n = 3$)

4.3.2.2 Pigments

Pigments were measured for each of the Antarctic diatom strains to see if there was any discernable effect of Se. The pigment data shows each of the diatom strains had higher chl *a* and total pigments when Se was present in the culture medium regardless of Se growth requirement (Figure 4.14). As discussed previously, *Thalassiosira antarctica* may have an Se growth requirement, unseen in the growth rate but displayed here.

Investigating which pigments were affected by different Se concentration (as percentage of total pigment per cell, to account for the higher levels of pigments in Se-replete cells) reveals diatoxanthin varied for all strains between Se treatments. Diatoxanthin is part of the xanthophyll cycle along with diadinoxanthin; the xanthophyll cycle involves the conversion of pigments from non-energy quenching forms to energy quenching forms. In diatoms at high light, diadinoxanthin is converted to diatoxanthin (by de-epoxidation) to reduce the amount of energy reaching the photosynthetic reaction centres. It is a protective mechanism to prevent photoinhibition. The light level at which these strains were grown (continuous 100 μmole photons m^{-2} s^{-1}) was slightly above saturating irradiance. Diatoms are reported to need low irradiance and light:dark cycle [71], so could be light-stressed during this experiment.

Iron deficiency has been shown to cause de-epoxidation of the xanthophyll cycle in terrestrial plants [72], and in *Phaeocystis* sp., diatoxanthin content increased while diadinoxathin remained similar under Fe-depleted conditions [73]. This appears to occur in our experiments, as levels of diadinoxanthin do not vary significantly. The ratio of diadinoxanthin to diatoxantion increased in all three diatom strains with higher Se in the growth medium (*Fragilariopsis* – 0.55 to 0.80; *Chaetoceros* – 1.35 to 6.60; and *Thalassiosira antarctica* – 0.75 to 2.24). This result suggests light stress is increased when no Se is added to the growth media. This fits well with the finding of decreased $rETR_{max}$ and E_k (half saturating light) observed for Se-depleted *E. hux* (Table 4.6).

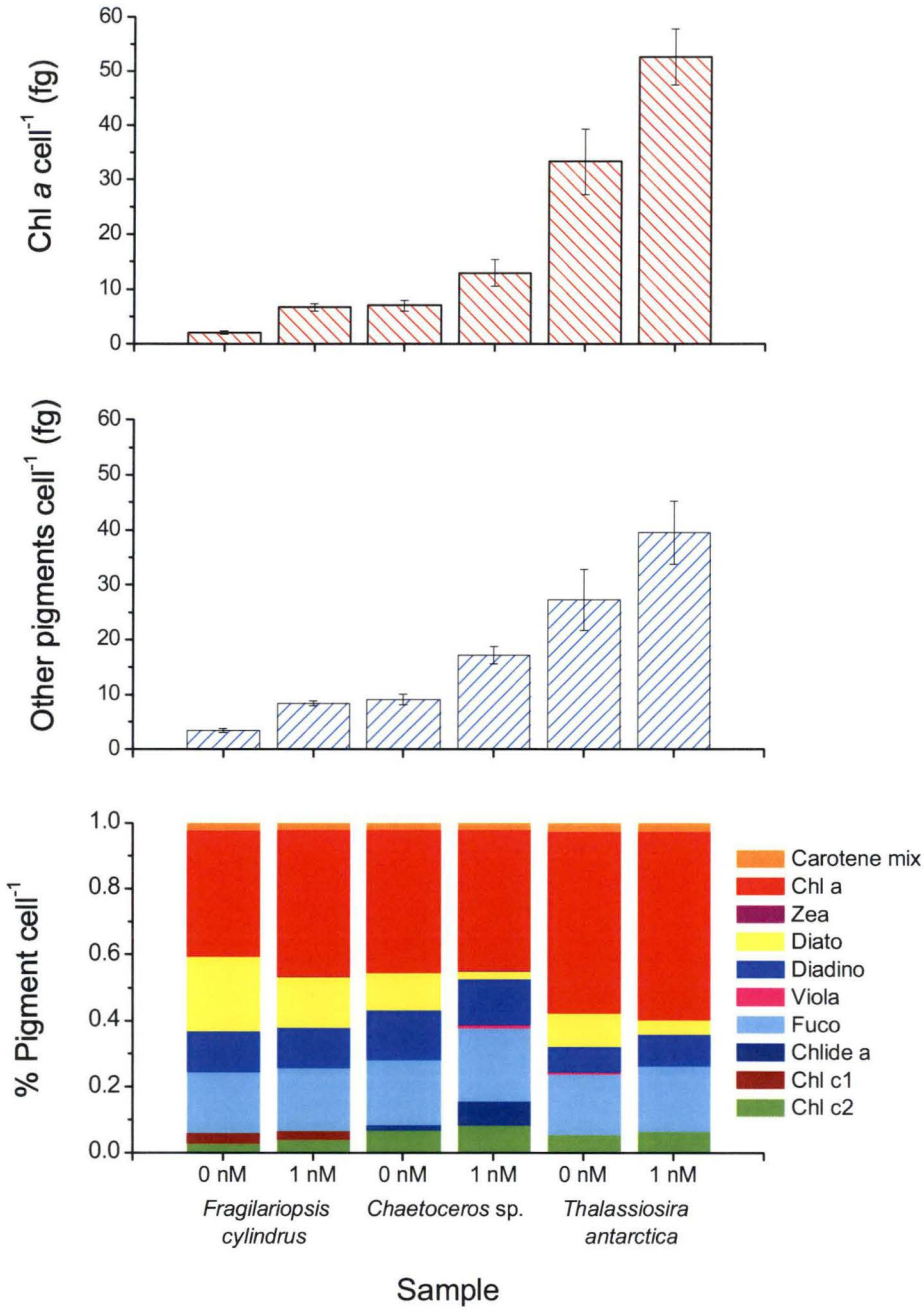

Figure 4.14. Pigments for the Antarctic diatom strains

Chl *c* – chlorophyll *c*; Chlide *a* – chlorophyllide *a*; Fuco – fucoxanthin; Viola – violaxanthin; Diadino – diadinoxanthin; Diato – diatoxanthin; Zea – zeaxanthin;

4.4 Discussion

Syn did not show any effects from the different Se concentrations on any parameter measured. If *Syn* has a Se requirement it is very low and is met by the background levels of Se in the medium (< 5 pM). It is more likely that *Syn* (at least the strain examined in this work) does not have a Se requirement.

E. hux has a definite Se requirement for growth. The exact concentration required for optimum growth is still undefined, but it is in the region of 0.05–1 nM Se(IV). This study did not investigate the ability of the cells to utilise other Se chemical forms. Previous work with *E.hux* had shown Se(IV) (as SeO_2 and Na_2SeO_3) to be effective from 1 nM Se whereas Se(VI) was not effective below concentrations of 1 μM [40].

The differences seen in *E. hux* photosynthetic parameters, where photosynthetic efficiency (F_v/F_m) was increased in Se-depleted cells (0 nM G3), suggest that short-term depletion of Se may be beneficial to cell health. This is supported by the add-back experiment, where both photosynthetic activity and growth were greatly enhanced.

For the Antarctic strains, two of the three diatoms showed a strong Se growth requirement. *Chaetoceros* sp. had a definite Se growth requirement which appeared to be met by 1 nM Se (we did not test whether it has a requirement for higher Se concentrations). *Fragilariopsis cylindrus* showed a slight Se growth requirement. It is unknown whether this requirement would be more evident in a second growth cycle when all intracellular Se supplies have been depleted. This is worthy of further investigation. *Thalassiosira antarctica*, did not show a growth requirement, even though there were changes evident in the pigments of the cells. Growing the cells for successive growth cycles may reveal a Se growth requirement that was met by intracellular Se in this experiment. Similarly, the growth of *Phaeocystis* sp. was not affected at any Se concentration tested here.

In general, there is a requirement for Se by the majority of diatoms from all regions of the ocean. Seven strains of *Chaetoceros* (this study and [18, 20]) and

six strains of *Thalassiosira* (this study and [18, 20]) have now been tested for Se growth requirement. Three of the seven *Chaetoceros* did not show a requirement, whereas only one of the *Thalassiosira* can be confirmed as not having a requirement. Our work on *Thalassiosira antarctica* can not conclusively rule out a growth requirement. Many of the previous growth studies have looked purely at growth rate and cell biomass. It would be interesting to investigate further to see if there are changes to photosynthetic parameters in those cells.

The increase in all pigments in the diatoms is an interesting finding. It is hard to say why there is an increase, although it may be due to the cells being replete, since increased pigment concentrations were seen in Antarctic sea ice diatoms between N-replete and N-depleted conditions [74]. This cannot be said definitively here because only two of the strains were shown to have a growth rate and biomass limitation.

What role Se plays in diatom cells needs to be further investigated. There is clearly a coupling between dissolved Se and Si in the oceans (as shown in Chapter 3 and previously reported [75, 76]). Whether this link is a result of interdependent requirement by diatoms for both of these elements has yet to be investigated.

4.5 Conclusions

This work has investigated the Se requirement of two temperate and four polar phytoplankton strains. We investigated the growth of the cells and, for the first time, changes in photosynthetic parameters following Se additions. Se was shown to affect the photosystem of species with a Se growth requirement, although the exact role it has in the phytoplanktonic cell is still unknown. The absolute biological requirement for Se was, thus, demonstrated in laboratory cultures, with varying requirements among different species.

4.6 References

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Chapter 5

A field study exploring phytoplankton community requirement for selenium

5.1 Introduction

The elemental requirements of phytoplankton vary across species and communities, and much remains unknown about the micronutrient requirements for a variety of trace elements, including selenium [1]. Laboratory culturing typically offers information on a single phytoplankton species under very controlled conditions. Such experiments do not replicate what is happening in the natural oceanic environment, where many phytoplankton species and other micro-organisms coexist and compete for the available nutrient supply. The role of the key micronutrient, iron (it has an essential role in photosynthesis and for the metabolism of nitrate [1, 2]) has been investigated in a number of ways for its effect on phytoplankton growth. In addition to laboratory culturing studies, utilising monocultures, there have been field investigations into the Fe requirements of the natural phytoplankton assemblage – artificial and natural *in-situ* fertilisation experiments (e.g. [3-5]) and deckboard incubations (e.g. [6-9]). A comparison of these two field methods reports that *in-situ* and ship-board incubations display the same trends, with the effects of Fe addition being enhanced in the bottle experiments [5]. The *in-situ* studies into Fe limitation on community structure have shown the effect of addition is not seen equally across the phytoplankton community, with larger phytoplankton size fractions, such as diatoms (8-20 μm), showing the greatest increase in biomass [5].

In-situ Fe experiments were originally trialled after results from deckboard incubation experiments showed increased growth when Fe was added to low Fe waters [10]. The *in-situ* experiments offer the chance for a longer, and more natural, investigation into the addition of Fe on the natural plankton assemblage. However, whilst there are many advantages to *in-situ* additions, these experiments are logistically very difficult and resource-consuming. Deckboard experiments provide an easier method to gain an insight into micronutrient requirements of phytoplankton communities.

5.1.1 Deckboard incubations

Deck incubations have been used previously to test for various macronutrient and metal/micronutrient limitations (or co-limitations) [7, 11-15]. Limitation is suggested by the elemental requirements of phytoplankton in laboratory cultures, combined with the measured concentration of the required element in regions of the ocean.

Iron is the most investigated element for phytoplankton requirements, with studies often investigating the addition of Fe in combination with macro- or micro-nutrients. Calculations of the half-saturation constant for growth (the concentration which supports half the maximum uptake rate, reflecting the ability of the phytoplankton cells to use low levels of required nutrients [16]) can provide insight into which phytoplankton species are limited in a region. Half-saturation constants vary between different phytoplankton species. For example, the values for half-saturation of Fe for a variety of diatoms were found to vary over three orders of magnitude [17], with small cells generally having the lowest values and Antarctic species having values at or above the measured dissolved Fe concentration [17].

Whilst Fe limitation has been proven and additions have shown clear enhancement of phytoplankton growth, the investigation of other micronutrients has not provided as clearly defined results. Zinc has been investigated in the high latitude oceans. In the Pacific sector of the Southern Ocean, where the measured bioavailable Zn concentrations suggest possible limitation, Zn additions did not stimulate phytoplankton growth [12]. Crawford and colleagues [18] report Zn additions in the Northeastern Subarctic Pacific Ocean to increase small diatoms and coccolithophores in comparison to control samples. They also see a shift in phytoplankton size when comparing combined Zn and Fe additions with only Fe additions, with an increase in the chlorophyll of the 0.2–5 μm fraction and a decrease in >20 μm fraction, compared to Fe addition alone.

Cobalt has also been added in deckboard experiments. In the Costa Rica up-welling region, when added with Fe, it was seen to increase chlorophyll to a greater extent than either metal added individually [13]. Any addition of Co was

seen to increase the numbers of cyanobacteria *Synechococcus*, which Fe alone did not do. Interestingly, the addition of vitamin B_{12}, which has Co as its metal centre, in experiments in the Ross Sea (Antarctica) showed no effect. However, Fe added alone increased growth, and B_{12} in combination with Fe caused even greater growth enhancement than single metal additions (in two of three sample sites) [15]. In this case, the site that did not show the enhancement had high levels of bacteria and archaea (thought to supply vitamin B_{12} to phytoplankton), and had a different initial phytoplankton community, with more sea-ice diatoms present. The samples with only vitamin B_{12} addition had decreased numbers of *Phaeocystis antarctica* relative to diatoms, while those samples with only Fe addition had increased cell numbers. This indicates that vitamin B_{12} additions, in combination with Fe, affect different phytoplankton species in different ways. This study also highlights how the initial community present can affect the observed results.

The effects of manganese have been studied intensively in the Atlantic sector of the Southern Ocean [9]. In one set of five experiments, daily Mn additions led to increased chlorophyll and a final particulate organic carbon (POC) concentration almost equal to that seen with Fe addition. The other four experiments showed more modest trends with a slight increase in chlorophyll and decreases in macronutrient concentrations, with POC relatively unchanged.

Other micronutrients are yet to be investigated for their effects on the natural phytoplankton assemblage using deckboard incubations, and even fewer studies have involved a sequence of additions. As was suggested by Crawford et al. [18] for Zn and Bertrand et al. [15] for vitamin B_{12}, these elements are thought to behave more subtly, influencing individual growth rates and community structure as a result of the varied requirements by different phytoplankton species. With Fe addition, if a site is initially limited, growth will ensue so long as there are sufficient nutrients. Other micronutrients, which are not essential to all phytoplankton, may only show results depending on initial community structure, and are likely to influence this structure disproportionately, favouring some phytoplankton species and enhancing their growth whilst others are unaffected.

Deckboard incubations have been criticised for the exclusion of large grazers from the incubations bottles. The exclusion of the larger grazers can result in higher biomass and chl *a*, which can be misinterpreted as a result of the macro- and/or micro-nutrient additions to the bottles [19]. It is a difficult question to determine what causes the increased biomass, however, this can be overcome by measuring the physiological state of the cells, as this will be unaffected by grazing effects [20].

5.1.2 This study

The study of Se speciation in the Southern Ocean (Chapter 3) showed the lowest surface water Se(IV) levels were found at the northern end of the transect, in temperate waters of the subtropical zone. This study region is not isolated from land for æolian and riverine or continental shelf inputs, so it could be expected to have higher concentrations because of these inputs. However, the region had higher biological productivity, possibly causing the observed decrease in Se concentrations. The laboratory culturing studies of two temperate phytoplankton species (Chapter 4) showed the coccolithophore *Emiliania huxleyi* to have a Se growth requirement, whilst cyanobacteria *Synechococcus* sp. did not. Previous reported studies have focussed on temperate species, so there is more information available on the Se growth requirements of various strains from these regions of the ocean (Chapter 4, Table 4.1). The laboratory culturing studies for Se have shown there to be a wide variety of species / strains that require Se. This growth requirement has not been linked to the natural environment, where Se(IV) levels are much lower than those used in the requirement studies (0.1 nM compared to ~1 nM or higher). The importance of Se on growth and phytoplankton community composition is yet to be demonstrated. Harrison et al. [21] calculated the half-saturation constant of Se for growth for four diatom strains (three coastal and one open ocean) to be ~1 nM for each of these species. They theorised that the growth rate of some phytoplankton species may be Se-limited in oligotrophic waters. This work investigated what discernable effects the addition of Se had on natural phytoplankton assemblages in the temperate ocean, in this case the Tasman Sea.

5.2 Characteristics of the Study Region

The Tasman Sea lies between Australia and New Zealand, extending northeast to the island archipelago of New Caledonia, Vanuatu and Fiji. Near the Australian coast, the East Australian Current (EAC) is a strong current, transporting waters from the tropics southward along the continental shelf. Along its path eddies peel off into the Tasman Sea (Figure 5.1). The Tasman Front (TF) is a continuation of the EAC; it is a strong eastward flow, at 33°–35°S, but is not a water mass boundary. Its path meanders across the Tasman Sea, to the northern tip of New Zealand, guided by a series of submarine ridges [22], ensuing from a coupling of the upper ocean with the bottom topography [23]. The Tasman Sea region is a low nutrient, low chlorophyll area, where the plankton community is dominated by the smaller size classes. It is possibly a nitrogen-limited region. The main aim of the fieldwork was to develop a nitrogen budget for the region.

There are a number of permanent warm-core eddies, associated with the extension of the Tasman Front flow, around the north and east coast of New Zealand ([23] and references cited within; Figure 5.1), which may vary in size and strength but are found in preferred locations, thought to be dictated by topography. Owing to few oceanographic surveys in the region, one of these eddies has only recently been formally identified – the Norfolk Eddy ([23]). Its formation is near the Norfolk Ridge, and it is centred in the Norfolk Basin.

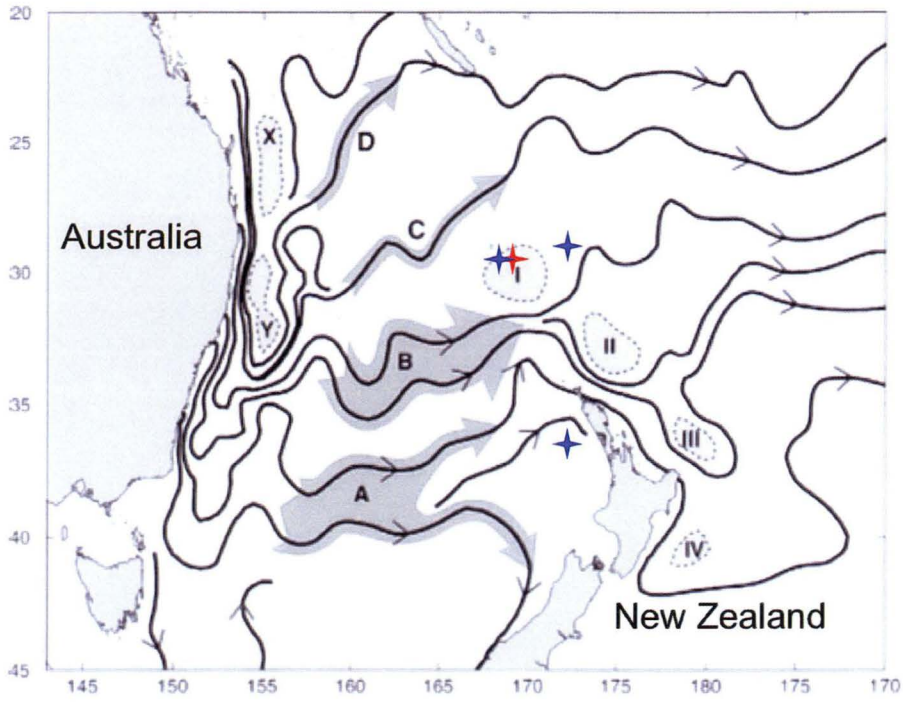


Figure 5.1 Illustration of the surface currents and eddies within the Tasman Sea. The four components of the separating East Australian Current (EAC) flow are designated A-D; B designates the flow associated with the Tasman Front. The four quasi-permanent eddies surrounding New Zealand are: I Norfolk Eddy; II North Cape Eddy; III East Cape Eddy; and IV Wairarapa Eddy. X and Y designate anti-cyclonic recirculation cells associated with the EAC [23]. Approximate locations of the study sites are indicated: COST – blue crosses; NCycle – red cross.

5.3 Methods

5.3.1 Study Area and Sampling

5.3.1.1 Water Sampling

Water samples were collected from the region in the late austral summer 2005 and 2006. Initial investigations were conducted in the northern Tasman Sea during February 2005, COST voyage (Characterising Oligotrophic Sub-Tropical waters), on *RV Tangaroa*. The COST voyage aimed to identify the limiting factors in the oligotrophic waters of the Tasman Sea. It occupied four stations – two north and

two south of the Tasman Front. Samples for Se were collected at three stations – two north of the Tasman Front (Station 1 – 30° 2' S 168° 44' E; Station 2 – 28° 43' S 171° 7' E) and one south of the Tasman Front (36° 36' S 170° 42' E). A large internal tide (12 h) was discovered, causing the thermocline and deep chlorophyll maximum to move by ~20 m, depending on the tide phase. This tide may have transported nutrients from the deeper waters into the euphotic zone.

On this voyage, samples were collected for Se, by Go-Flo bottles on Kevlar line, into acid-cleaned 250-ml HDPE bottles and were stored in the dark unacidified at room temperature. Samples were not acidified as they were collected by a third party. The correct acid was unavailable onboard. Samples were acidified upon arrival in Australia; this was a maximum of three weeks after collection. Samples were analysed (after a maximum storage period of 6 months), in the shore-based laboratory. The stability of Se speciation in samples is discussed in Chapter 2 and samples are shown to be stable under these conditions.

More detailed investigations and the deckboard incubations were conducted on the NCycle voyage during March-April 2006 on *RV Tangaroa* (TAN0603). The NCycle voyage aimed to characterise nitrogen pathways, including supply, fixation, mixing and fate, in this region. The voyage hoped to constrain the nitrogen cycle in the waters of the region as the FeCycle voyage had achieved for Fe in the subantarctic waters south of New Zealand [24]. A single site was investigated because of time constraints. It was selected based upon predicted current speeds using sea surface height altimetry data supplied by Melissa Bowen (NIWA). The chosen site was 29° 30'S 170° 15'E, north of the Tasman Front, where current speeds were ~0.1 m s^{-1}. A thermistor chain drifter buoy was deployed to provide a reference point for sampling throughout the 13-d station occupation.

For most deckboard studies, surface seawater is collected and placed into containers (1-24 l), and various additions are made to investigate the desired parameters. To ensure the water sampled is uncontaminated, it needs to be collected by various specialised trace-metal sampling methods (e.g. [25]). Commonly used methods for discrete sampling include GoFlo or Niskin-X bottles

suspended on Kevlar hydroline or on a trace-metal-clean rosette, while underway sampling can be achieved using a clean sub-surface towfish. Collection of samples whilst underway means there may be some small variability between samples, and therefore samples should be homogenised before trace metal addition. Samples for various parameters are measured at the start of the experiment and then at regular time points through the experiment, which can last up to 16 days. The length of the experiment is chosen to suit the region in which the samples were collected For example, in colder regions, phytoplankton have slower growth rates, and therefore, a longer period is needed to see possible effects of the additions.

On this voyage, a towed torpedo-shaped fish (deployed to the starboard side) was used for surface water sampling. It pumped water from ~1 m depth while *RV Tangaroa* was steaming at 1-12 knots [26]. To prevent trace-metal contamination, the fish was epoxy coated, with all LDPE tubing (acid cleaned) and an all-Teflon diaphragm pump. The water was pumped into a Class-100 clean air laboratory where it was subsampled. Samples were collected for the deckboard incubations and dissolved Se analysis.

Depth samples were collected for dissolved Se from both 10-l Niskin samplers deployed on a standard conductivity-temperature-depth (CTD) rosette, and from 5-l Niskin-1010X samplers deployed on a new autonomous 1018 intelligent rosette system specially adapted for trace metal work (General Oceanics) [27]. All samples were collected into 250-ml acid-cleaned HDPE bottles.

For the trace-metal clean cast, the rosette was lowered to depth and slowly raised to the surface. The bottles were automatically closed at the appropriate depth, whilst the rosette was still moving, using a pre-programmed pressure sensor. The trace-metal rosette sampled to a maximum depth of 400 m. Once on-board, the sampling bottles were plastic-bagged and transferred to a Class-100 clean air laboratory for processing. The bottles were pressurised (with N_2) and samples filtered (0.2 μm cartridges, Sartobran®). Additional samples were taken unfiltered, to compare with filtered samples, as samples from the standard CTD (on this voyage and I9S, Chapter 3) were not filtered.

5.3.1.2 Deckboard incubations

Two incubation experiments were conducted during the voyage, each for the duration of four days. The first experiment investigated the effect of Fe and Se, whilst the second experiment looked at N and Se (Table 5.1). The second experiment investigated N as other incubation experiments on board showed the region to be N-limited.

Table 5.1 Deckboard incubation experiment treatments

	Treatments			
Incubation 1	Control	+Fe	+Se	+Fe+Se
Incubation 2	Control	+N	+Se	+N+Se

Additions to the incubations were at the following levels:

Fe as Fe(III)	2 nM	Fe atomic absorption standard solution, 1000 $\mu g\ l^{-1}$ in 1% HNO_3 (Sigma-Aldrich)
Se as Se(IV)	2 nM	SeO_2 (Spex Industries)
N as NO_3	2 nM	$NaNO_3$ (Sigma-Aldrich; Chelex-extracted to remove possible metal contamination)

The samples for the deckboard incubations were pumped directly from the towfish into the 4-l polycarbonate bottles used in the experiment. The samples were not pre-filtered to exclude grazing organisms and all treatments were run in duplicate.

To minimise risk of contamination, the bottles were tightly closed, the caps sealed with Parafilm and taped before being placed in the incubators. The incubators were supplied with flowing surface seawater to maintain the *in-situ* temperature

(~20-21°C) and shaded to ~40% ambient light levels. The bottles were randomly placed in the incubators to minimise any bias due to their position.

Incubated bottles were subsampled each 24 h for the next 4 days. The sampling occurred at the same time each day, in the early evening as this was when the experiments were first placed in the incubators. The duration of the experiments was chosen to allow two sets of incubations to be run during the site occupation, thus allowing investigations into questions raised by the first set of incubations. In addition, this experimental design allowed for sufficient volume to be taken for subsampling each day to measure the desired parameters, whilst minimising the possibility of bottle effects having an influence on results.

For subsampling, the bottles were transferred to the clean laboratory where samples were collected daily for flow cytometry counts, nutrients (phosphate, nitrate, nitrite and ammonium), Se(IV), and fast repetition rate fluorometry (FRRF). The limited volume of water available meant that repeat measurements were not possible.

Samples for Se(IV) were filtered (0.2 μm, Sartobran® cartridge filter) and acidified, and stored at ambient temperature in the dark. Analysis was conducted on return to the shore-based laboratory, within 4 months of sampling, following the methods outlined in Chapter 2. Phytoplankton and bacterial abundance were determined by flow cytometry following the procedures of Hall et al. [28]. Samples were analysed immediately upon sampling, with Trucount™ beads (50 μl) being added to each sample as a tracer. Bacteria, *Synechococcus*, *Prochlorococcus* and eukaryotic phytoplankton were distinguished in each sample. Nutrient measurements were made immediately upon sampling. Ammonium determination was by diffusion (of ammonia) through a Teflon membrane into a fluorescent reagent with fluorometric detection. Nitrate, nitrite and phosphate were determined using a three-channel 'nanomolar-level' analytical system with colorimetric analytical techniques, using waveguide capillary cells of 2-m path length [29, 30]. Limits of detection and precision for this method are reported in Woodward and Rees [30], and for this voyage were

nitrate – 1 ± 0.1 nM; nitrite – 0.5 ± 0.03 nM; and phosphate – 2 ± 0.2 nM [31]. Silicate was not measured routinely.

Photosynthetic capacity of the phytoplankton community was measured by FRRF. All samples were dark-acclimated for 20-40 min prior to measurement in the dark chamber of the FRRF.

5.4 Results and discussion

5.4.1 COST results

The COST voyage was the preliminary investigation of the region. It provided the information necessary for the following NCycle voyage. Briefly, chl *a* was typically 0.4-0.6 μg l^{-1}, with 62-89% of chl *a* concentration in the <2 μm size fraction. This size fraction was seen to dominate at all stations and all depths. Nitrate and phosphate concentrations were below LOD in the surface water (N ≤0.07 μM; P ≤0.03 μM), only detectable below 90 m (except at the southern most station, 50 m). The values then rose steadily with depth, to maximum values of ~35 μM N and ~2.5 μM P.

Se concentrations were low in the surface waters, with increases with depth (as seen in Chapter 3) (Figure 5.2). The highest values for organic Se were seen in the station south of the Tasman Front. These were the highest values seen throughout the field studies undertaken for this thesis, with a maximum value of 0.17 nM, compared to the highest value seen on the I9S transect of 0.09 nM. The maximum value of 0.17 nM accounted for 27.8% of Se at that station depth, with Se(IV) being 0.06 nM.

The precision of the Se measurements for inorganic Se is ±0.02 nM; and for organic Se is ±0.04 nM.

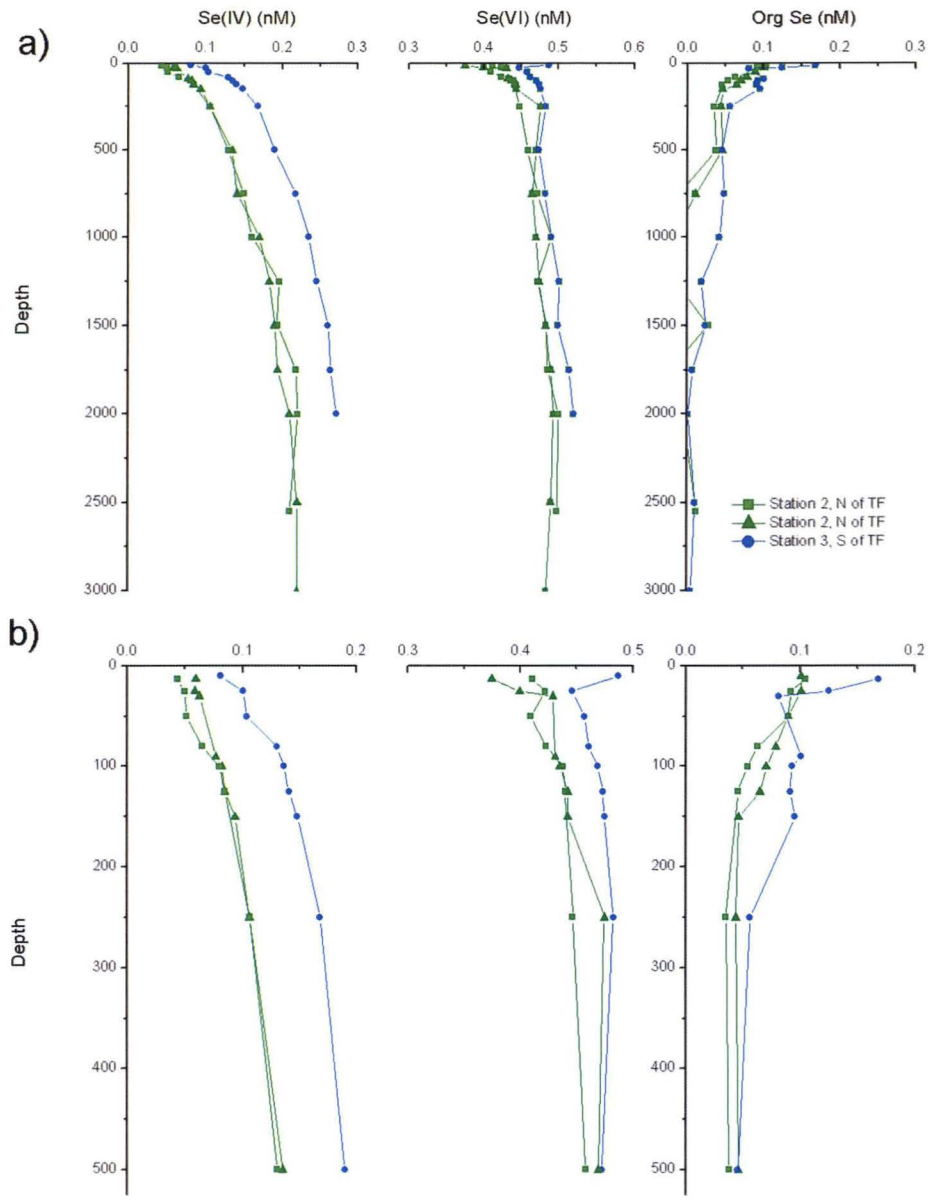


Figure 5.2 Concentration of Se at three stations during the COST voyage a) full water column samples; b) top 500 m samples NB different scale for concentrations between a and b, Station 1 and 2 were located north of the Tasman Front; Station 3 was south of the Tasman Front.

5.4.2 NCycle

The NCycle voyage was a more thorough approach to the investigation of nitrogen cycling in this region. This voyage had the advantage of nanomolar nutrient measurements to properly quantify the levels of nitrate, nitrite, ammonium and phosphate in the surface waters, which had been below the detection limit of traditional nutrient analytical techniques used on the COST voyage.

An important source of nitrogen to N-limited regions is from nitrogen fixing phytoplankton such as *Trichodesmium*. A bloom of *Trichodesmium* occurred during this voyage, resulting in nitrogen fixation from the atmosphere.

5.4.2.1 Filtered vs. unfiltered samples

The Se data for the Southern Ocean transect presented in Chapter 3, was obtained from analysis of unfiltered samples, with an appropriate justification contained in that chapter. In this investigation, two sets of samples—unfiltered and filtered—were collected from the trace-metal clean rosette at selected casts. For this work, and the comparison between traditional and clean sampling techniques (5.4.2.2), care was taken to ensure samples were collected at similar times of day to avoid changes in upper water column concentrations because of the large internal tide. The comparison of Se values between the two will show if significant amounts of Se are present in the particulate phase that is measured by the HG-CT-AFS technique. This is not expected, owing to the low biomass of the sampling region.

Previous reported Se studies have used various approaches for filtration. These include the use of 0.4-μm polycarbonate filter membranes inline for surface samples and 0.45-μm filter cartridge for depth samples [32]; underway surface samples were 0.4-μm cartridge filtered but for depth profile samples, filtration was unreported [33]; and others recorded no information on filtration, presumably because there was no filtration done.

The comparison of filtered and unfiltered samples showed there to be no variation in Se concentration due to filtration (Figure 5.3). In areas with high productivity and biomass, filtration may be necessary for those samples but otherwise it appears to not be required. This confirms that the results obtained for I9S transect are valid (as presented in Chapter 3).

5.4.2.2 Traditional vs. clean sampling techniques

The comparison between unfiltered and filtered samples was conducted to see if there was a difference in Se concentration as a result of particulate material in the samples. A further comparison test was done to test for contamination by traditional sampling methods; it used samples collected from the trace-metal-clean rosette and the standard CTD rosette. The length of Kevlar line onboard, meant the trace-metal-clean rosette could only be deployed to 400 m. The samples were unfiltered. For some elements, it is essential that samples are collected by specialist 'clean' techniques. Se is not as easily contaminated and has historically been sampled by both traditional [32, 34] and clean [33] techniques.

The two sampling methods did not appear to affect Se concentration in the samples in this instance (Figure 5.3). There was some small variability in the upper water column samples but this was most likely because of the interval between the different samplings. These differences were not significant and may even be due to experimental variability.

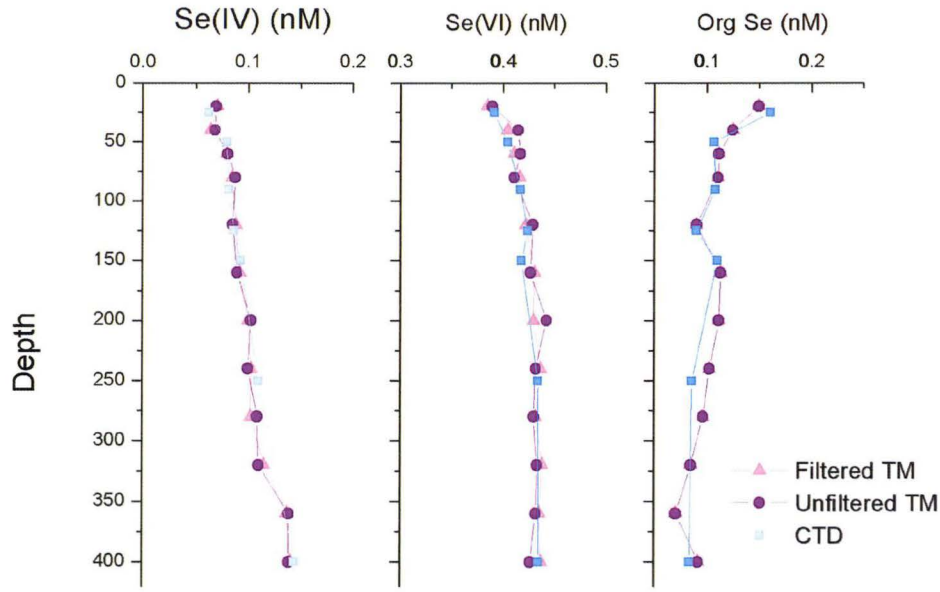


Figure 5.3 Selenium concentrations found in filtered and unfiltered samples from the trace-clean rosette and unfiltered samples by traditional sampling techniques

5.4.2.3 Deckboard incubations

5.4.2.3a Flow cytometry

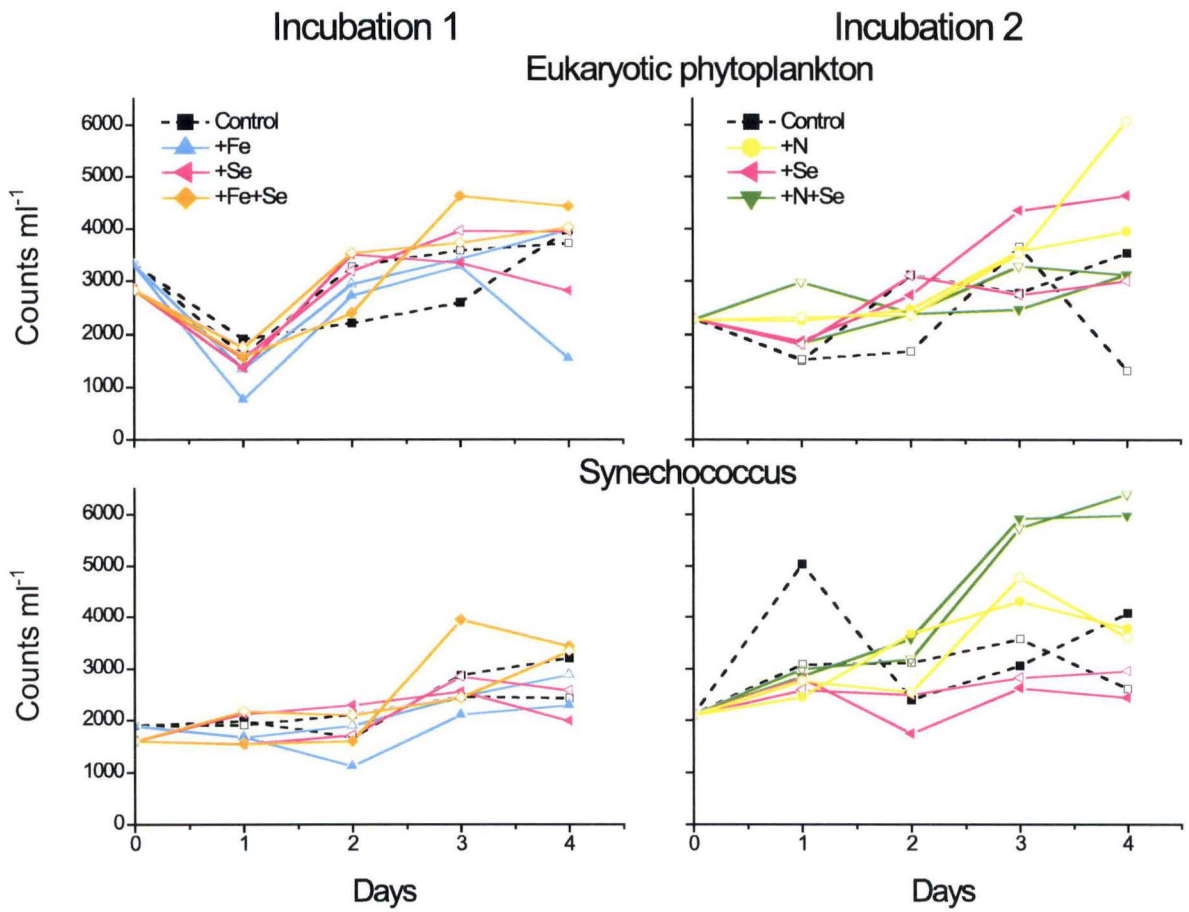
Incubation experiment 1, that of Fe and Se addition (Table 5.1), showed no difference in numbers of the different phytoplankton types between the control samples and any of the treated samples (Figure 5.4). Other incubations conducted onboard (data not shown) at the same time as the first incubation experiment, revealed there to be a primary limitation of growth because of low nitrogen and a possible secondary limitation because of low phosphate in this region. Phosphate only became limiting upon addition of N, which stimulated growth until P was exhausted. These experiments showed no responses in any treatments other than when N was added. The concentration of nitrogen at the start of incubation experiment 1 was 11.8 nM (Figure 5.5b); phosphate, was not as low, at 50.3 nM (Figure 5.5a).

In incubation experiment 2, nitrogen was added to overcome the N-limitation. In this experiment, no change was seen for the eukaryotes during the experiment (Figure 5.4a). The larger cells might have needed longer to show growth changes, possibly due to a lag phase or simply because of the relatively slower growth rate.

There was a small increase in *Synechococcus* for the +N+Se treatment (Figure 5.4b). This was not seen for the single addition of either N or Se. The laboratory monoculture experiments (Chapter 4) did not show *Synechococcus* to have a Se growth requirement. Plausible reasons why the combination of N and Se caused an increase, while N alone did not, are considered below.

Prochlorococcus numbers were greatly increased in the N treatment (Figure 5.4c). This increase was also seen in the +N+Se treatment, but to a much smaller degree. It is not known why the increase was smaller when there was the same level of N added. The results for the N treatment could have arisen from *Synechococcus* being out-competed by *Prochlorococcus* in the mixed community incubations. Whereas, the large increases of *Synechococcus* in the +N+Se treatment, at the apparent expense of *Prochlorococcus,* might be explained by the Se addition having a negative effect on *Prochlorococcus*; no reports were found for the laboratory study of Se and *Prochlorococcus,* so this effect of Se is unknown, and remains to be tested.

Bacteria appear to be unaffected by any of the treatments of +N+Se, with no variation from the control (Figure 5.4d). The experiments were not filtered so grazers may have had a role in limiting bacterial increases.


Incubation 1
Incubation 2
Eukaryotic phytoplankton
Control
+Fe
+Se
+Fe+Se
Control
+N
+Se
+N+Se
Synechococcus
Counts ml-1
Counts ml-1
0
1000
2000
3000
4000
5000
6000
0
1
2
3
4
Days
Days

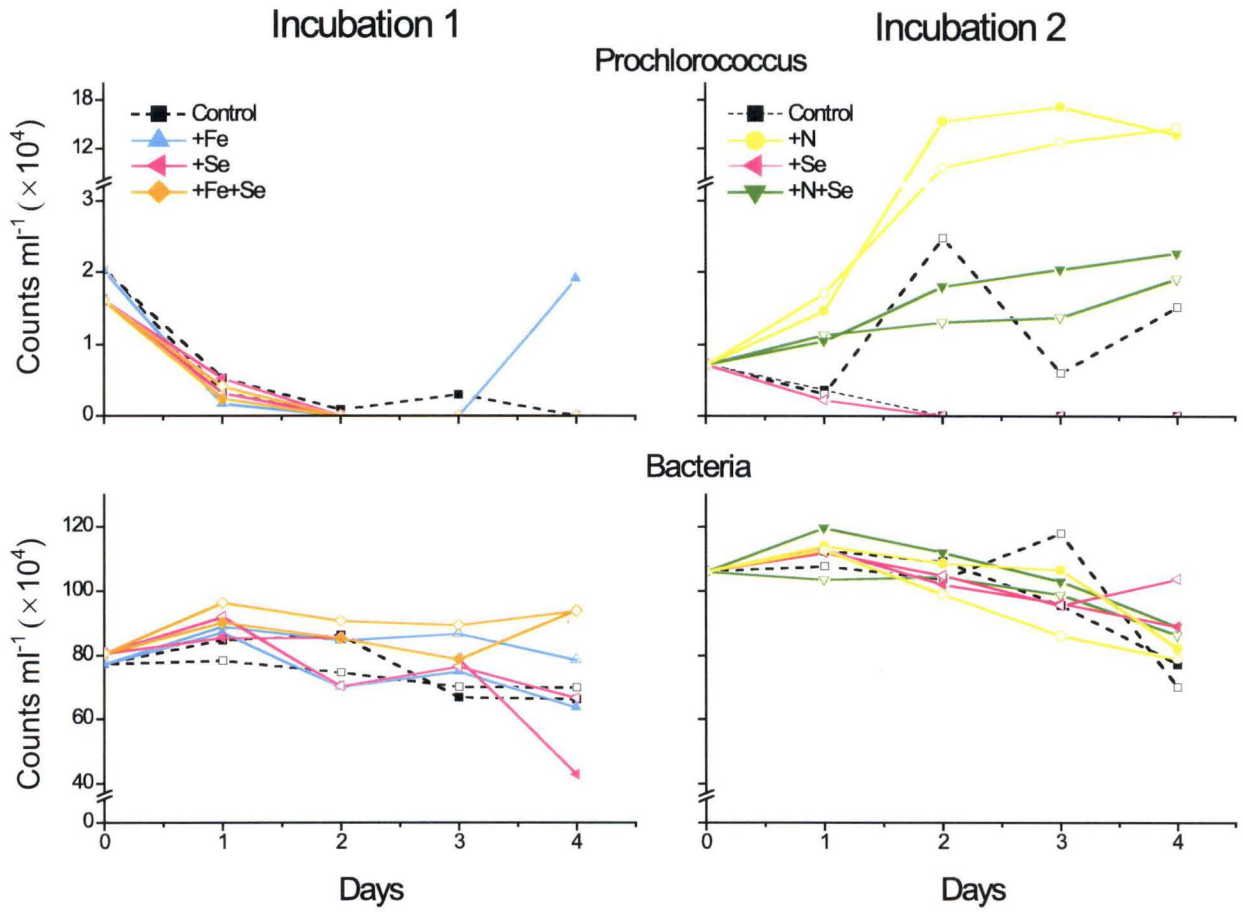


Figure 5.4 Cell numbers in the incubation experiments a) Eukaryotic phytoplankton; b) *Synechococcus*; c) *Prochlorococcus*; d) Bacteria

5.4.2.3b Nutrient concentrations

There was no difference in the nutrient concentrations between the different treatments, except for when N was added. Silicate was not routinely measured, but its concentration was high (<18 μM) and in excess in this region, and thus not considered to limit growth. In the first experiment, N limited growth so there was no drawdown of the other macronutrients.

In the second experiment, when N was added (+N and +N+Se treatments), there was a decrease in nitrate (from the addition level of 5 μM to ~2 μM; data not shown for +N and +N+Se) and an increase in nitrite over the course of the experiment (Figure 5.5c). The control sample showed higher nitrate levels at days 1 and 2. The reasons behind the observed increase are unknown. There is large variability in the measurement on day 2, only one of the control bottles had an elevated value. Both of the control bottles had increased cell counts for a cyanobacteria, one was elevated for *Synechococcus* at day 1 and the other for *Prochlorococcus* at day 2 (Figure 5.5b, c). The reason for these elevated counts is unknown.

There was no increase in the ammonium concentration over the 4-d experiment (Figure 5.5d). There was a slight drawdown of phosphate with the N treatment (from background levels of ~60 nM to ~41 nM for both +N and +N+Se; compared to ~53 nM for non-N treated, Figure 5.5a), if the experiment had run longer than 4 days, this might have become greater. This incubation ran over a short time frame, other studies, typically colder regions where productivity rates are lower, have run up to 16 days [11]. A study in the northeast Pacific, investigating Fe and Zn, ran for 8 d with nutrient drawdown only becoming clear at day 4 [14].

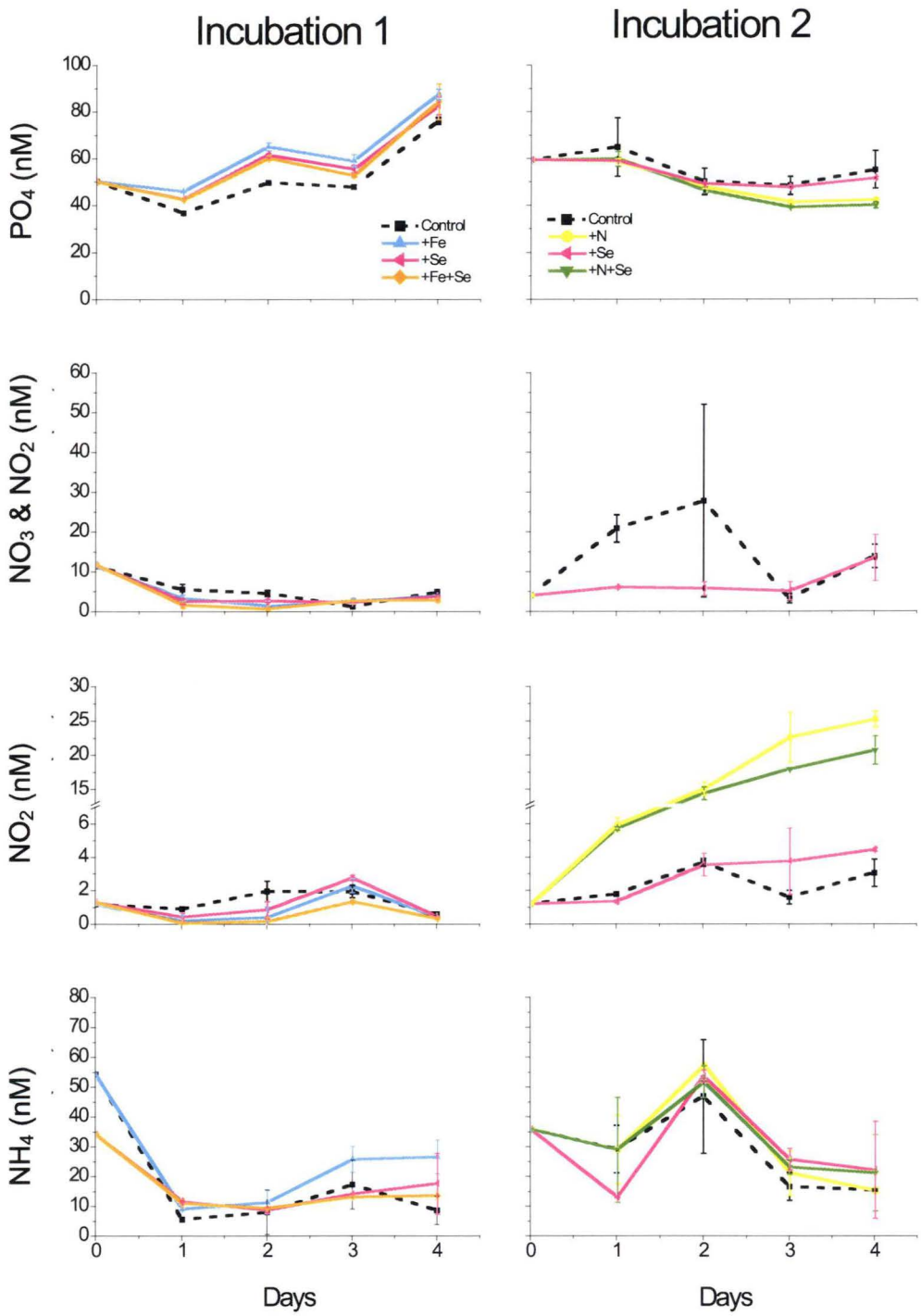


Figure 5.5 Nutrient concentrations for each treatment over the 4-d experiment a) Phosphate, b) Nitrate and Nitrite, c) Nitrite, d) Ammonium.

NB: NO_3&NO_2 data is not shown for Incubation 2, treatments +N and +N+Se

5.4.2.3c Photosynthetic activity by fast repetition rate fluorometry

Quantum yield (F_v/F_m) is a measure of the efficiency of the photosystem of phytoplankton cell, or more simply a measure of how healthy the cells are. Higher quantum yields indicate more efficient light capture. Quantum yield was discussed in Chapter 4, with data presented there obtained from a pulse amplified modulation (PAM) fluorometer. PAM fluorometry lacks the sensitivity needed for the study of phytoplankton in the open, oligotrophic ocean, whereas FRRF can be used with very low chlorophyll *a* concentrations, <0.1 mg m^{-3} [35]. Although these systems work in different ways, they both measure the photosystem efficiency of the cells. The data for quantum yield for the two methods is related but in a non-linear fashion [35]; and through the use of a non-linear function, it is possible to compare data [36].

Previous field experiments have shown quantum yield to be an early indicator of limitation in the cell, and it can be seen before a reduction in cell numbers. Boyd and colleagues [37] report that F_v/F_m is the "most representative index of iron concentrations on algal physiology", as seen for the phytoplankton bloom during an *in-situ* Fe fertilisation experiment in the subarctic Pacific Ocean (measured by FRRF). They saw an increase in the quantum yield within 24 h of Fe addition, with steady values for 11 d, followed by a decrease as the Fe supply was exhausted, demonstrating Fe addition had increased the photosynthetic capacity of the cells.

F_0, the background fluorescence of the cells, gives a representation of the concentration of all photosynthetic pigments present in the cells, not just chlorophyll *a* [38]. It shows the light absorbing capacity of the cells. Maximum values of 0.65 can be expected for F_v/F_m in nutrient-replete laboratory cultures [39]. Coastal samples have been seen to approach 0.60, as have *in-situ* Fe fertilisation experiments [40], whereas F_v/F_m rarely exceeds 0.5 in open ocean samples [41].

The F_v/F_m values in the first incubation experiment were all higher than seen in the second experiment (Figure 5.6a), but no difference could be seen between any of the treatments. In the second experiment, N addition, alone and with Se, caused increases in F_v/F_m values. The Se only addition initially showed no effect, but by T4 the increases were comparable to those of N addition. Higher values indicate cells are less stressed. The values observed here were not near the maximum that can be expected, although oligotrophic waters have been seen to have lower values with significant variability between different ocean areas ([42] and references within). It is unknown if this increase at T4 is representative of the effect of Se. It may be that cells take longer to respond to this micronutrient addition. Further experiments need to be conducted to confirm that this is the case.

F_0 did vary between treatments, with an increase to almost double the other treatments when N was added (Figure 5.6b). F_0 is the direct measure of fluorescence of dark-adapted cells, compared to F_v/F_m which is a measure of the change between initial and maximum fluorescence. An increase in F_0 shows that there are more cells, thus higher fluorescence emission.

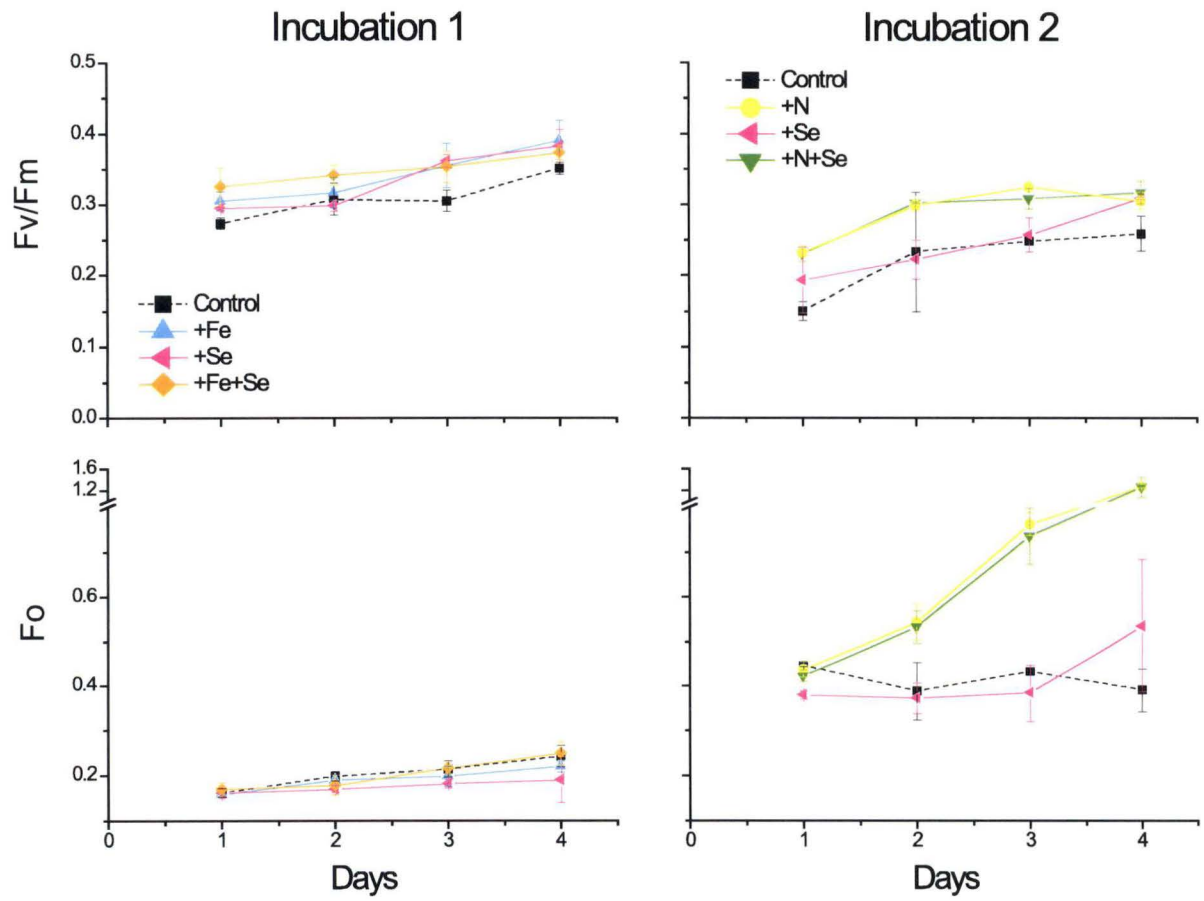


Figure 5.6 a) Quantum yield and b) fluorescence measurements by fast repetition rate fluorometry for deckboard incubation experiments

5.5 Conclusions

The incubation experiments showed small increases in photosynthetic efficiencies of the cells for the addition of N and Se. The addition of N also showed a slight drawdown of P. Increases in picoplankton were seen for N additions.

The results for the incubations show that, in the short time of these incubations, Se(IV) addition did not significantly affect growth rates or photosynthetic parameters. The length of the experiments needs to be considered, as to whether it was enough time for shifts in the community structure. The presence of surplus cellular Se may also have been sufficient not to allow Se-depletion to become evident in the limited time of the experiments. The depletion of the additional Se may have been the result of Se being taken up into a pool of intracellular Se, surplus to requirements, as has been reported in laboratory culturing work [43]. No measures were made of intracellular Se to confirm this.

Extending the duration of the incubations, under the current design, may lead to more information on Se effects but it introduces the complication of bottle effects. A much more detailed experimental plan would need to be in place to determine which parameter was causing any observed changes. Either having larger incubation bottles, to allow for daily subsampling; or have smaller bottles that are sacrificially sampled (needing more bottles to allow for sufficient time points over the course of the experiment) could overcome these problems.

The site chosen was an N-limited region. During the course of the study a *Trichodesmium* bloom occurred. The presence of these nitrogen-fixing phytoplankton meant that there was an external source of N to these waters. This additional source of N may have alleviated some of the N limitation. However, these experiments did not show Se to influence growth alone or in combination with N. The primary characteristic of the region is N limitation.

Even though there was no discernable response from Se addition in these experiments, they still provide interesting information. Before an experiment like

this is attempted again, consideration should be given to responses that we did not look for here. The use of other analytical tools, such as pigment analysis or microscope counts – to provide more information of what species are present through the course of the experiment – could be used for greater understanding of possible effects of the various additions. Site selection and timing of the experiments are two important points. Four days does not appear long enough in this region to see if Se was having an effect. The increase in quantum yield, for Incubation 2 at T4, with +Se to the level seen in +N treatments is a tantalising data point. If it is a true value, Se may have an important role in this region. Unfortunately, the experiments concluded and have left that question unanswered.

A longer term study, such as that done by Sherrard et al [32], where the Se concentrations and site characteristics over different seasons were measured, may provide additional insight into the natural cycling of Se in a particular region and allow a more informed choice for the appropriateness of addition experiments. Further work is required, to overcome the differences between laboratory culturing and natural community deck incubations, before the role Se has in influencing ecosystem health becomes clear.

5.6 References

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Chapter 6

Conclusions & future work

6.1 Conclusions and future work

Oceanographic studies of Se have previously involved the collection of samples at sea with subsequent measurement upon return to a shore-based laboratory. The method developed in this work allows the measurement of Se species onboard a research vessel, dispensing with the need for storage of samples. Analysis is by use of one compact instrument, with various pretreatments to determine the three Se species (Chapter 2). The stability of the Se species influenced the choice of pretreatment conditions. The optimised methods have been tested to demonstrate that they provide unambiguous results. The analytical methodology was validated both in the shore-based laboratory (Chapter 2) and at sea (Chapter 3).

The AFS instrument is also capable of measuring the volatile Se species (such as dimethylselenide and dimethyldiselenide) if samples are analysed immediately by a purge-and-trap technique [1] – this was not investigated in this work, but would represent an interesting extension. Data on volatile Se species have only been reported in the eastern Mediterranean Sea [2], and the North Atlantic Ocean [3], where it was shown that the Se gases were closely linked to the S gases.

Since Se(IV) is both a minor and bio-assimilated species in the surface ocean, and it is the directly measured form in HG-CT-AFS, it was anticipated that preconcentration could be required in oligotrophic waters. A literature review indicated that co-precipitation would be a suitable method, and in particular, using lanthanum hydroxide (Chapter 2). This was confirmed through experiments, although preconcentration proved unnecessary for the seawater samples measured over the course of this work. Subsequently, Tang et al. [4] have published an on-line preconcentration system for AFS using the same strategy.

The oceanographic study during the CLIVAR I9S transect in the Indian sector of the Southern Ocean generated a new and extensive Se dataset for this ocean region (Chapter 3). The lowest values of Se were seen in the subtropical waters at the northern end of the transect, increasing southward to the continent (corroborated by work in the Tasman Sea, Chapter 5). The relation between Se and macronutrients was not as clear in this study as had been shown previously.

Further Se data are required to investigate if the biogeochemical links between Se and macronutrients in the Southern Ocean are different to those in other areas of the ocean. The ratio of Se(IV) to Se(VI), previously reported as a possible tracer for water masses, showed deep water values close to unity, higher than previously reported. Very few deep-water profiles exist for Se and the comparison between deep-water ratios has not been for individual water masses. With the collection of more deep samples it would be possible to compare like with like and see how the Se(IV)/Se(VI) ratio is related to water-mass properties and age.

Seasonal changes in the concentration and speciation of Se, in response to phytoplankton blooms, is an interesting area that has only recently been studied. Sherrard et al. [5] reported data indicating seasonal control on Se distributions, but over a single year for two stations separated by the Subtropical Front. The limited studies on Se need to be expanded to fully understand Se distribution in the ocean and its interactions with chemical and physical features. Results should be intercalibrated to ensure that published analytical methods are comparable. Since most of the data for Se in the ocean are from the GEOSECS era in the 1970s, it would be beneficial to include Se measurements on a limited number of research voyages that form part of the new GEOTRACES program.

The laboratory culturing study for Se requirement confirmed results from previous studies [6, 7], and built on these by exploring other regulatory factors (Chapter 4). *Emiliania huxleyi* was shown to have an absolute growth requirement for Se, while *Synechococcus* sp. did not show a requirement for, nor any observed influence of, the metalloid. The investigation into Se requirement of four Antarctic phytoplankton species—the first study of this kind—revealed varying Se requirements. The Se requirement of phytoplankton appears to be very species-dependent.

This work is the first to report on the effect that Se has on the photosynthetic systems of phytoplankton. Cellular pigments were seen to change when no growth change was detected. The effect of Se is greater than a growth requirement; it appears to play a role in the photosystems of phytoplankton cells, possibly influencing the ability to deal with excessive irradiance and reactive oxygen

species. Se is a known essential component of glutathione peroxidase, an enzyme that protects cells from oxidative damage. Whilst this enzyme has been identified in only a few phytoplanktonic cells, Se may play a similar role (via different chemistry) in many phytoplanktonic cells.

Studies into the effect of Se on a phytoplankton community assemblage, in a low Se region, did not provide conclusive results (Chapter 5). Experiments such as this have successfully shown Fe limitation [8], but results for other micronutrients are less clearly defined [9-11]. For these elements and Se, additional thought may need to be applied in determining the best experimental design, duration, and which are the best monitored parameters to discern their subtle influence over surface ocean biogeochemistry.

The role of Se in the phytoplankton cell is a major question that still needs answering. Its requirement by diatoms, and role in enzymatic processes for silicate formation in sponges [12], could mean Se has a role in Si skeleton formation of diatoms, although with them it is a non-enzymatic process. The need for Se by the most abundant of the coccolithophores (*E. hux*), leads to the question of a role in the formation of the carbonate skeleton or coccoliths. Work by Fabry [13] showed *E. hux* to have a Se growth requirement, however, this was strongest in the non-calcifying strains. Further investigation is needed to determine if Se does have an effect on silicification and calcification rates. Another function of Se may be in cell structure and cell division. When *E. hux* was grown in Se-depleted media, the cells reached a very large size (~135 μm^3 compared to ~58 μm^3 for Se-replete cells) as they appeared to be unable to divide. It has been shown that *E. hux* have larger cell size under stressful conditions (e.g., elevated irradiance [14]) so the increase cell size seen in this work may simply be a result of stress. However, Se deficiency has been reported to cause changes in cell shape and size for diatom *Thalassiosira pseudonana* [15], and the blockage of cell division resulting in enlarged cells. This cellular size increase was also seen in a range of diatoms under Se-depleted conditions [7]. The role of Se in maintaining cell structure needs to be investigated more fully, with identification of Se-containing proteins and their function being an important study.

Whatever is the role of Se in the cell, demonstrating its effect on phytoplankton in the natural environment will be a more difficult task. In the Southern Ocean, Se does not appear to be limiting under current conditions. The subtropical regions are more likely to be areas where Se might have a limiting or co-limiting role. This role is likely to be a subtle one, influencing community structure, since the laboratory culturing studies have shown Se requirement to differ greatly among species. The chemical composition of the environment as well as physical characteristics will also have an effect on the role of Se, as Se uptake has been shown to vary with light [16] and nutrient concentrations [17].

The role and determination of organic Se is the other important question to arise from this work. As discussed in 3.3.10, reported values for organic Se differ greatly and it is unknown what compounds are being measured. Work on laboratory cultures, if done with sufficient volume and phytoplankton cell concentrations, could allow the measurement of the different compounds present in the culture media, or possibly even intracellularly. It has been shown that chromatographic analytical techniques are capable of separating and detecting many organic Se compounds [18], although extremely sensitive detection would be required in this instance. Knowledge of the nature of the organic Se complexes produced by various phytoplankton species in mono-cultures would allow development of experiments to test for these species in the natural environment. Recently, marine proteomics studies have discovered seven new Se protein families [19]. The Se proteins have been found, it is now up to us to determine what their role is.

Anther topic of interest, which has not been touched on in this study, is the link between Se and S in the oceanic cycle. As was mentioned previously, the oceanic volatile Se species have been linked to volatile S species. Se has been linked to S in terrestrial studies, with studies on the two elements in sediments [20] and with bacteria reducing Se in the same way as S [21]. In the ocean, S is present as a major ion, SO_4^{2-}, with minor interactions with biology. The other minor forms of S, reduced forms and / or organic forms are the ones of greater interest. The areas of study that would be interesting are the volatile species and the organic species. One of the major volatile Se species is an Se-S compound [3]. The two elements

are clearly linked, but the manner in which this occurs would be of great interest. It might be a way in which to gain a greater understanding of the nature of the organic Se fraction in the ocean, and how it cycles.

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Appendix 1

CLIVAR I9S selenium dataset

station	lat	long	depth	Se(IV)	Se(VI)	Total Se	org Se	botsalt	phos	nit	sil	do	temp	Chl a
2	-34.82	115.00	16.03	0.0946	0.3242	0.5128	0.0940	35.8270	0.13	0.0	2.6	236.8	20.091	-
2	-34.82	115.00	40.87	0.1046	0.3413	0.5215	0.0756	35.8213	0.11	0.0	2.6	237.8	20.019	-
2	-34.82	115.00	60.20	0.1278	0.3308	0.5349	0.0762	35.7990	0.15	0.0	2.7	232.7	19.504	-
2	-34.82	115.00	81.44	0.1168	0.3556	0.5572	0.0848	35.8101	0.15	0.3	2.8	229.8	19.355	-
2	-34.82	115.00	101.47	0.1479	0.3485	0.5477	0.0514	35.8114	0.14	0.4	2.8	233.1	19.318	-
2	-34.82	115.00	128.84	0.1400	0.3625	0.5514	0.0489	35.8280	0.14	0.4	2.8	234.3	18.986	0.21675
7	-35.65	115.01	11.36	0.1681	0.4257	0.6362	0.0425	35.8783	0.10	0.0	2.5	237.0	19.499	-
7	-35.65	115.01	51.13	0.1424	0.3843	0.6155	0.0888	35.6979	0.13	0.0	2.0	251.5	17.065	-
7	-35.65	115.01	101.91	0.1754	0.3926	0.6475	0.0795	35.4589	0.33	2.1	1.8	250.7	14.589	-
7	-35.65	115.01	151.66	0.1434	0.4185	0.6523	0.0904	35.2149	0.42	4.2	1.8	254.5	13.048	-
7	-35.65	115.01	200.85	0.1543	0.4229	0.6489	0.0718	35.0945	0.54	6.3	2.0	254.0	12.136	-
7	-35.65	115.01	300.42	0.1621	0.4430	0.6536	0.0485	34.8319	0.85	11.2	3.1	253.6	10.420	-
7	-35.65	115.01	401.96	0.1974	0.4575	0.6845	0.0295	34.7223	1.01	14.5	4.3	249.6	9.599	-
7	-35.65	115.01	551.10	0.1784	0.4734	0.6737	0.0219	34.5948	1.27	18.7	6.8	237.4	8.693	-
7	-35.65	115.01	700.55	0.1670	0.4764	0.6834	0.0400	34.4768	1.67	24.9	16.2	207.1	7.140	-
7	-35.65	115.01	850.55	0.1714	0.4920	0.7220	0.0586	34.3831	2.01	29.9	30.6	200.9	5.077	-
7	-35.65	115.01	1002.15	0.1945	0.4815	0.7129	0.0369	34.3942	2.21	32.1	46.2	193.0	3.883	-
7	-35.65	115.01	1302.23	0.2129	0.5012	0.7493	0.0352	34.5132	2.39	34.1	69.2	171.4	3.035	-
7	-35.65	115.01	1699.28	0.2733	0.4943	0.7592	-0.0083	34.6572	2.37	33.9	88.7	164.5	2.633	-
7	-35.65	115.01	2100.84	0.2590	0.4831	0.7533	0.0111	34.7160	2.32	33.1	101.3	172.9	2.271	-
7	-35.65	115.01	2501.00	0.2478	0.4795	0.7303	0.0029	34.7306	2.29	32.7	109.1	181.3	1.919	-
7	-35.65	115.01	2899.57	0.2549	0.4847	0.7386	-0.0010	34.7321	2.28	32.1	114.2	188.4	1.697	-
7	-35.65	115.01	3250.58	0.2699	0.4727	0.7403	-0.0024	34.7308	2.27	32.1	116.5	194.2	1.523	-
7	-35.65	115.01	3500.25	0.2802	0.4521	0.7379	0.0056	34.7282	2.26	32.4	118.9	196.3	1.429	-
7	-35.65	115.01	3999.55	0.2695	0.4815	0.7457	-0.0052	34.7223	2.27	32.1	121.3	201.0	1.290	-
7	-35.65	115.01	4502.33	0.2732	0.4973	0.7802	0.0096	34.7135	2.27	32.1	123.6	209.6	1.059	-
7	-35.65	115.01	4996.79	0.2639	0.4992	0.7721	0.0090	34.7074	2.27	32.3	126.2	213.7	0.966	-
7	-35.65	115.01	5137.56	0.2793	0.5196	0.8032	0.0043	34.7070	2.30	32.4	126.7	-	0.966	-

station	lat	long	depth	Se(IV)	Se(VI)	Total Se	org Se	botsalt	phos	nit	sil	do	temp	Chl a
15	-39.70	115.00	10.68	0.1390	0.3567	0.5823	0.0867	35.2353	0.26	1.0	0.7	65.3	15.851	-
15	-39.70	115.00	51.59	0.1304	0.4016	0.5794	0.0474	35.4569	0.24	0.6	0.9	270.5	14.393	-
15	-39.70	115.00	99.82	0.1235	0.3916	0.5925	0.0773	35.3504	0.41	4.0	2.1	252.7	13.496	-
15	-39.70	115.00	150.08	0.1416	0.4004	0.5817	0.0397	35.1246	0.57	6.5	2.2	256.0	12.144	-
15	-39.70	115.00	200.65	0.1453	0.3891	0.5786	0.0441	35.1063	0.59	7.1	2.4	260.8	11.857	-
15	-39.70	115.00	300.78	0.1549	0.4265	0.6019	0.0204	34.9194	0.75	9.9	3.0	263.2	10.829	-
15	-39.70	115.00	400.46	0.1605	0.4315	0.6232	0.0312	34.7429	0.98	13.9	4.3	254.3	9.771	-
15	-39.70	115.00	501.39	0.1788	0.4272	0.6160	0.0101	34.6717	1.07	15.7	4.9	254.7	9.280	-
15	-39.70	115.00	598.84	0.1786	0.4314	0.6188	0.0088	34.6289	1.15	17.2	5.5	252.3	8.982	-
15	-39.70	115.00	699.74	0.1678	0.4408	0.6329	0.0242	34.5619	1.34	20.2	8.2	234.4	8.401	-
15	-39.70	115.00	850.72	0.1838	0.4522	0.6437	0.0077	34.4560	1.77	26.3	18.0	205.1	6.845	-
15	-39.70	115.00	1000.62	0.1831	0.4818	0.6641	-0.0008	34.3705	1.99	30.4	29.0	205.5	5.066	-
15	-39.70	115.00	1249.60	0.2703	0.4704	0.7084	-0.0323	34.5094	2.26	33.5	51.3	189.6	3.583	-
15	-39.70	115.00	1500.06	0.2677	0.4726	0.7315	-0.0088	34.5279	2.37	35.1	70.5	169.0	3.025	-
15	-39.70	115.00	2000.23	0.2742	0.4944	0.7630	-0.0055	34.6805	2.32	33.7	88.1	171.3	2.536	-
15	-39.70	115.00	2502.51	0.2792	0.4879	0.7750	0.0079	34.7357	2.21	32.1	94.0	187.2	2.134	-
15	-39.70	115.00	2999.73	0.2769	0.4886	0.7712	0.0057	34.7414	2.18	32.0	102.2	197.6	1.720	-
15	-39.70	115.00	3501.82	0.2732	0.4901	0.7763	0.0129	34.7290	2.18	31.7	112.0	207.1	1.301	-
15	-39.70	115.00	4001.13	0.2839	0.5085	0.7932	0.0009	34.7098	2.23	32.2	122.9	215.9	0.897	-
15	-39.70	115.00	4810.27	0.2942	0.5495	0.8594	0.0157	34.7041	2.25	32.5	126.9	217.1	0.856	-
20	-42.51	114.99	10.55	0.1152	0.3640	0.5286	0.0494	34.7096	0.66	9.0	1.8	278.9	12.497	0.39009
20	-42.51	114.99	55.51	0.1259	0.3514	0.5178	0.0406	34.6950	0.81	10.6	3.3	289.0	10.436	0.49558
20	-42.51	114.99	100.42	0.1353	0.3858	0.5214	0.0003	34.7477	0.82	11.8	3.4	271.9	10.016	0.11202
20	-42.51	114.99	154.06	0.1512	0.3746	0.5389	0.0131	34.7221	0.87	13.0	4.0	274.2	9.740	0.01916
20	-42.51	114.99	201.06	0.1714	0.3872	0.5816	0.0230	34.7119	0.90	13.3	4.2	273.4	9.579	-
20	-42.51	114.99	302.18	0.2253	0.4208	0.6312	-0.0150	34.7047	0.95	14.0	5.1	271.9	9.479	-
20	-42.51	114.99	402.26	0.1892	0.3998	0.6342	0.0452	34.6525	-	-	-	-	9.414	-
20	-42.51	114.99	549.40	0.1900	0.4298	0.6515	0.0317	34.6613	1.04	15.9	5.4	261.6	9.186	-
20	-42.51	114.99	700.40	0.2408	0.4313	0.6723	0.0001	34.5416	1.37	20.7	8.9	239.3	8.287	-

station	lat	long	depth	Se(IV)	Se(VI)	Total Se	org Se	botsalt	phos	nit	sil	do	temp	Chl a
20	-42.51	114.99	848.72	0.2497	0.4737	0.7188	-0.0045	34.4430	1.67	26.1	16.6	-	6.890	-
20	-42.51	114.99	999.87	0.2737	0.4649	0.7213	-0.0174	34.5216	-	-	-	-	5.466	-
20	-42.51	114.99	1149.49	0.2656	0.4807	0.7437	-0.0026	34.3353	2.10	32.3	34.3	212.4	4.226	-
20	-42.51	114.99	1351.70	0.3105	0.5017	0.7933	-0.0189	34.3982	2.27	34.7	51.8	194.7	3.353	-
20	-42.51	114.99	1548.66	0.3007	0.5293	0.8213	-0.0087	34.4932	2.32	36.2	64.0	180.3	2.894	-
20	-42.51	114.99	1801.05	0.3311	0.5322	0.8765	0.0132	34.6036	2.34	35.3	74.6	174.8	2.629	-
20	-42.51	114.99	2100.30	0.3466	0.5345	0.8731	-0.0081	34.6929	2.23	34.0	81.2	183.1	2.428	-
20	-42.51	114.99	2499.37	0.4017	0.5412	0.9479	0.0050	34.7477	2.09	31.8	83.5	197.6	2.151	-
20	-42.51	114.99	2901.56	0.3753	0.5411	0.9011	-0.0153	34.7478	2.14	32.1	97.3	203.2	1.722	-
20	-42.51	114.99	3700.19	0.4001	0.5489	0.9341	-0.0149	34.7121	2.24	33.3	121.0	214.2	0.942	-
20	-42.51	114.99	4401.10	0.3901	0.5520	0.9219	-0.0202	34.7045	2.24	33.4	125.5	216.6	0.828	-
28	-46.51	115.04	10.68	0.1559	0.3959	0.6450	0.0933	34.2182	1.00	14.1	1.1	290.4	9.791	0.28557
28	-46.51	115.04	62.19	0.1566	0.3792	0.6296	0.0937	34.4558	0.89	12.4	1.8	287.5	9.836	0.29344
28	-46.51	115.04	91.52	0.1461	0.4002	0.6373	0.0910	-	1.08	14.0	2.0	291.3	8.649	0.35112
28	-46.51	115.04	121.21	0.1489	0.4070	0.6403	0.0845	34.4311	1.13	15.1	3.7	284.8	8.497	0.13653
28	-46.51	115.04	149.17	0.1960	0.4100	0.6491	0.0431	34.4915	1.12	15.5	4.6	281.1	8.652	0.07050
28	-46.51	115.04	201.21	0.2191	0.4414	0.7210	0.0605	34.5207	1.13	15.9	5.4	277.1	8.629	-
28	-46.51	115.04	302.34	0.2084	0.4617	0.7196	0.0495	34.4723	1.17	16.9	6.0	278.1	8.251	-
28	-46.51	115.04	401.00	0.2365	0.4638	0.7649	0.0646	34.4413	1.25	18.5	7.0	267.9	7.949	-
28	-46.51	115.04	603.03	0.2973	0.4589	0.8039	0.0477	34.3870	1.60	24.2	14.0	235.4	6.748	-
28	-46.51	115.04	699.77	0.2977	0.4796	0.8298	0.0526	34.3778	1.77	26.9	19.2	220.5	6.000	-
28	-46.51	115.04	848.62	0.3435	0.4975	0.8890	0.0480	34.3135	1.98	30.1	26.7	222.8	4.650	-
28	-46.51	115.04	1000.38	0.4392	0.4899	0.8920	-0.0371	34.3227	2.14	32.2	36.7	214.7	3.821	-
28	-46.51	115.04	1201.59	0.4332	0.4900	0.9218	-0.0015	34.4093	2.28	33.8	51.7	193.6	3.262	-
28	-46.51	115.04	1400.97	0.4356	0.5056	0.9430	0.0017	34.4948	2.33	34.4	62.9	182.1	2.882	-
28	-46.51	115.04	1602.88	0.4481	0.5295	0.9901	0.0125	34.5809	2.31	34.0	69.9	180.2	2.657	-
28	-46.51	115.04	1899.83	0.4672	0.5200	0.9793	-0.0079	34.6752	2.20	32.7	74.8	184.7	2.478	-
28	-46.51	115.04	2200.44	0.5062	0.5260	1.0057	-0.0264	34.7310	2.13	31.4	79.1	193.8	2.254	-
28	-46.51	115.04	2802.05	0.4836	0.5002	0.9519	-0.0320	34.7467	2.13	31.3	96.9	204.6	1.673	-

station	lat	long	depth	Se(IV)	Se(VI)	Total Se	org Se	botsalt	phos	nit	sil	do	temp	Chl a
32	-48.46	115.03	20.79	0.2004	0.4031	0.6439	0.0404	34.1522	1.18	16.3	1.4	296.9	8.430	0.26374
32	-48.46	115.03	86.57	0.1847	0.4211	0.6710	0.0652	34.1917	1.24	16.7	1.9	297.1	7.752	0.33113
32	-48.46	115.03	101.46	0.1902	0.4387	0.6698	0.0409	34.2446	1.30	17.1	3.4	293.0	7.475	0.32768
32	-48.46	115.03	201.36	0.2217	0.4530	0.7050	0.0303	34.2898	1.41	20.2	7.3	277.6	7.046	0.04736
32	-48.46	115.03	301.49	0.2322	0.4742	0.7420	0.0356	34.2111	1.51	22.1	10.0	282.0	6.036	-
32	-48.46	115.03	399.04	0.2566	0.4700	0.7516	0.0250	34.2888	1.71	25.3	15.7	246.7	5.845	-
32	-48.46	115.03	501.55	0.2569	0.4912	0.7902	0.0421	34.3012	1.87	27.7	21.0	232.9	5.221	-
32	-48.46	115.03	600.06	0.2997	0.4800	0.8258	0.0461	34.3120	1.98	29.3	26.5	225.6	4.665	-
32	-48.46	115.03	704.05	0.3438	0.4860	0.8747	0.0449	34.2765	2.09	31.0	32.7	228.0	3.801	-
32	-48.46	115.03	799.61	0.3766	0.4878	0.8908	0.0265	34.3049	2.18	32.3	38.9	218.9	3.514	-
32	-48.46	115.03	1200.80	0.4387	0.5285	0.9744	0.0072	34.5073	2.33	34.3	66.3	181.9	2.818	-
32	-48.46	115.03	1603.50	0.4583	0.5200	0.9931	0.0148	34.6576	2.24	32.9	75.8	183.4	2.485	-
32	-48.46	115.03	2004.22	0.4969	0.5370	1.0199	-0.0140	34.7329	2.14	31.3	80.9	195.1	2.199	-
32	-48.46	115.03	2502.56	0.4923	0.5458	1.0399	0.0018	34.7508	2.11	30.7	92.7	204.3	1.777	-
32	-48.46	115.03	3201.44	0.5100	0.5671	1.0620	-0.0152	34.7259	2.20	31.8	114.0	210.1	1.202	-
32	-48.46	115.03	4019.50	0.4820	0.5610	1.0417	-0.0013	34.7149	2.24	32.1	120.0	216.3	1.043	-
35	-49.99	115.05	11.44	0.1877	0.3972	0.6508	0.0659	33.9510	1.38	20.9	1.9	310.8	6.462	-
35	-49.99	115.05	82.58	0.1691	0.4137	0.6613	0.0786	33.9813	1.58	22.3	4.6	317.3	4.545	-
35	-49.99	115.05	101.48	0.1813	0.4407	0.6841	0.0620	34.0187	1.58	22.7	5.9	310.9	4.586	-
35	-49.99	115.05	199.55	0.2038	0.4568	0.7298	0.0691	34.0488	1.69	25.4	14.3	301.0	4.050	-
35	-49.99	115.05	299.61	0.2013	0.4890	0.7599	0.0696	34.1416	1.89	28.6	22.5	267.4	3.745	-
35	-49.99	115.05	402.23	0.2252	0.4816	0.7260	0.0192	34.2035	2.04	30.7	30.3	245.9	3.414	-
35	-49.99	115.05	500.21	0.2594	0.5020	0.8006	0.0393	34.2777	2.17	32.4	40.5	223.4	3.101	-
35	-49.99	115.05	600.76	0.3388	0.5245	0.8699	0.0065	34.3549	2.27	34.3	51.0	205.2	2.857	-
35	-49.99	115.05	700.53	0.3351	0.5369	0.8681	-0.0039	34.4236	2.31	34.4	59.0	192.9	2.759	-
35	-49.99	115.05	850.74	0.3530	0.5452	0.8948	-0.0035	34.5066	2.31	34.4	66.4	183.7	2.635	-
35	-49.99	115.05	1001.04	0.3769	0.5414	0.9369	0.0185	34.5877	2.29	33.7	72.4	180.9	2.520	-
35	-49.99	115.05	2401.19	0.5171	0.5693	1.0638	-0.0226	34.7366	2.15	31.2	106.2	207.4	1.393	-
35	-49.99	115.05	3852.45	0.4776	0.5492	1.0306	0.0038	34.6962	2.26	32.5	127.1	220.3	0.628	-

station	lat	long	depth	Se(IV)	Se(VI)	Total Se	org Se	botsalt	phos	nit	sil	do	temp	Chl a
37	-51.01	115.07	12.60	0.2361	0.4002	0.7052	0.0689	33.9020	1.55	25.3	2.3	328.8	4.431	-
37	-51.01	115.07	50.59	0.1797	0.3994	0.6700	0.0909	33.9053	1.57	24.9	2.4	328.4	4.285	0.19118
37	-51.01	115.07	85.65	0.1693	0.4361	0.6796	0.0742	33.9362	1.72	25.6	6.3	335.0	2.949	0.23113
37	-51.01	115.07	141.07	0.1756	0.4453	0.6862	0.0653	33.9743	1.84	27.2	18.3	335.4	1.892	0.06750
37	-51.01	115.07	200.54	0.1960	0.4545	0.6854	0.0349	34.0318	1.97	30.0	30.1	318.7	1.316	-
37	-51.01	115.07	304.37	0.1882	0.4495	0.7013	0.0636	34.2217	2.20	33.4	45.9	248.7	2.021	-
37	-51.01	115.07	401.19	0.2183	0.4782	0.7267	0.0303	34.3713	2.31	34.7	58.8	206.2	2.284	-
37	-51.01	115.07	499.40	0.2456	0.4917	0.7384	0.0012	34.4521	2.34	35.5	66.2	192.7	2.276	-
37	-51.01	115.07	601.67	0.2305	0.5185	0.7519	0.0028	34.5462	2.30	34.7	71.1	182.8	2.423	-
37	-51.01	115.07	700.73	0.2578	0.5054	0.7725	0.0093	34.5866	2.30	34.6	74.2	181.6	2.315	-
37	-51.01	115.07	848.76	0.2590	0.5369	0.7949	-0.0010	34.6499	2.25	33.4	77.6	183.0	2.269	-
37	-51.01	115.07	1003.53	0.2739	0.5214	0.8079	0.0126	34.6886	2.20	32.8	80.3	186.3	2.170	-
37	-51.01	115.07	1201.47	0.3180	0.5289	0.8520	0.0050	34.7290	2.13	31.5	81.3	194.6	2.116	-
37	-51.01	115.07	1403.02	0.3614	0.5417	0.8815	-0.0216	34.7465	2.09	30.9	85.0	200.2	1.954	-
37	-51.01	115.07	1604.04	0.3887	0.5336	0.9048	-0.0175	34.7514	2.09	30.9	93.9	203.4	1.804	-
37	-51.01	115.07	1800.62	0.4144	0.5275	0.9308	-0.0111	34.7467	2.10	31.0	94.2	206.6	1.605	-
37	-51.01	115.07	2301.18	0.3861	0.5113	0.9206	0.0233	34.7272	2.17	31.8	110.9	210.6	1.149	-
37	-51.01	115.07	2596.00	0.3860	0.5239	0.9194	0.0095	34.7153	2.21	32.4	115.5	214.2	0.916	-
37	-51.01	115.07	3201.21	0.4107	0.5433	0.9682	0.0142	34.6935	2.27	33.0	129.7	223.7	0.462	-
37	-51.01	115.07	4027.55	0.4807	0.5522	1.0056	-0.0273	34.6856	2.28	32.8	133.0	226.8	0.366	-
41	-53.20	115.00	345.99	0.3290	0.4815	0.8723	0.0619	34.4132	-	-	-	202.6	2.032	-
41	-53.20	115.00	398.15	0.3635	0.4885	0.8900	0.0380	34.4895	2.33	35.5	71.6	183.8	2.246	-
41	-53.20	115.00	501.92	0.3823	0.4902	0.9075	0.0349	34.5701	2.25	34.6	75.0	180.3	2.283	-
41	-53.20	115.00	700.94	0.3795	0.4879	0.8897	0.0223	34.6603	2.17	33.5	79.6	183.2	2.220	-
41	-53.20	115.00	899.39	0.3747	0.4892	0.8865	0.0226	34.7123	2.12	32.0	82.7	190.7	2.102	-
41	-53.20	115.00	1500.94	0.3788	0.4800	0.8823	0.0235	34.7453	2.07	31.0	93.5	204.3	1.659	-
41	-53.20	115.00	2200.07	0.4188	0.5227	0.9319	-0.0096	34.7229	2.15	31.8	112.4	212.3	1.047	-
41	-53.20	115.00	2800.39	0.4209	0.5234	0.9500	0.0057	34.6950	2.20	32.6	125.7	220.6	0.545	-
41	-53.20	115.00	3982.26	0.4803	0.5469	1.0247	-0.0025	34.6819	-	32.8	136.4	229.7	0.259	-

station	lat	long	depth	Se(IV)	Se(VI)	Total Se	org Se	botsalt	phos	nit	sil	do	temp	Chl a
42	-53.82	114.98	15.85	0.2552	0.4007	0.7189	0.0631	33.8359	1.55	24.8	5.4	324.6	4.866	0.13898
42	-53.82	114.98	50.51	0.2197	0.4326	0.7275	0.0752	33.8398	1.56	24.9	5.4	326.3	4.705	0.34416
42	-53.82	114.98	80.25	0.2420	0.4518	0.7320	0.0381	33.9374	1.79	26.3	14.0	341.4	2.205	0.45108
42	-53.82	114.98	151.43	0.2371	0.4432	0.6905	0.0102	33.9753	1.92	28.8	26.0	331.7	1.289	0.12052
42	-53.82	114.98	200.50	0.2602	0.4575	0.7814	0.0637	34.0802	2.05	31.1	36.7	295.6	1.404	-
42	-53.82	114.98	252.06	0.2852	0.4700	0.7898	0.0346	34.1962	2.18	33.0	47.7	254.6	1.708	-
42	-53.82	114.98	302.67	0.2791	0.4792	0.8311	0.0727	34.3084	2.27	34.3	57.3	223.8	1.919	-
42	-53.82	114.98	351.12	0.3290	0.4662	0.8694	0.0742	34.4101	-	-	-	198.7	2.237	-
46	-56.20	114.99	16.65	0.2426	0.4571	0.7581	0.0583	33.8294	1.43	24.8	3.6	329.0	4.380	0.24555
46	-56.20	114.99	51.08	0.2561	0.4818	0.7601	0.0223	33.8324	1.48	25.1	4.8	329.7	4.158	0.31735
46	-56.20	114.99	80.93	0.2668	0.4664	0.7520	0.0187	33.8554	1.79	26.9	14.4	335.6	2.723	0.39254
46	-56.20	114.99	149.80	0.3313	0.4667	0.7831	-0.0149	33.9340	1.89	28.5	22.1	333.9	1.609	0.06966
46	-56.20	114.99	201.59	0.3193	0.4682	0.7917	0.0042	34.0369	2.05	31.6	33.5	304.1	1.311	-
46	-56.20	114.99	249.70	0.3262	0.4917	0.8278	0.0098	34.1921	2.20	34.8	46.5	254.2	1.752	-
46	-56.20	114.99	300.31	0.3117	0.5096	0.8664	0.0451	34.3141	2.29	35.3	57.0	218.2	2.006	-
46	-56.20	114.99	398.05	0.3185	0.5358	0.9027	0.0484	34.4376	2.36	35.9	66.5	192.4	2.247	-
46	-56.20	114.99	499.35	0.4071	0.5421	0.9603	0.0110	34.5287	2.35	35.7	72.2	180.1	2.342	-
46	-56.20	114.99	699.46	0.4171	0.5426	0.9967	0.0370	34.6333	2.26	34.1	76.8	180.8	2.332	-
46	-56.20	114.99	850.03	0.4602	0.5550	1.0300	0.0148	34.6782	2.24	33.3	80.6	184.3	2.189	-
46	-56.20	114.99	1002.16	0.5219	0.5389	1.0548	-0.0060	34.7115	2.18	32.7	82.4	190.1	2.116	-
46	-56.20	114.99	1602.40	0.4907	0.5499	1.0292	-0.0114	34.7477	2.11	31.5	92.9	204.2	1.690	-
46	-56.20	114.99	2502.79	0.4590	0.5540	1.0326	0.0196	34.7143	2.22	32.7	116.6	213.2	0.909	-
46	-56.20	114.99	3000.24	0.4713	0.5475	1.0063	-0.0125	34.6949	2.25	33.4	125.0	221.5	0.532	-
46	-56.20	114.99	4584.00	0.4559	0.5565	1.0320	0.0195	34.6711	2.27	33.4	138.5	238.7	0.027	-
50	-58.63	114.98	141.52	0.2925	0.4591	0.7641	0.0125	33.9833	1.97	29.4	39.7	346.2	-0.765	0.05931
50	-58.63	114.98	202.19	0.2905	0.4702	0.7730	0.0123	34.3061	2.29	34.1	60.2	228.8	1.455	-
50	-58.63	114.98	301.66	0.3169	0.4812	0.8404	0.0423	34.4895	2.38	35.2	73.8	182.7	2.168	-
50	-58.63	114.98	403.99	0.3450	0.4881	0.8652	0.0321	34.5828	2.32	34.5	79.2	175.9	2.226	-
50	-58.63	114.98	502.66	0.3882	0.4947	0.8802	-0.0027	34.6236	2.36	33.8	81.1	177.8	2.210	-

station	lat	long	depth	Se(IV)	Se(VI)	Total Se	org Se	botsalt	phos	nit	sil	do	temp	Chl a
50	-58.63	114.98	600.87	0.4076	0.4974	0.9011	-0.0039	34.6595	2.24	33.4	83.0	180.8	2.149	-
50	-58.63	114.98	752.92	0.4215	0.5120	0.9386	0.0051	34.6998	2.19	33.2	86.0	186.7	2.081	-
50	-58.63	114.98	1051.68	0.4509	0.5333	1.0096	0.0253	34.7382	2.10	31.0	90.1	196.6	1.903	-
50	-58.63	114.98	1151.90	0.4851	0.5291	1.0179	0.0037	34.7431	2.09	30.7	95.9	198.9	1.828	-
50	-58.63	114.98	1600.56	0.4700	0.5472	1.0047	-0.0126	34.7412	2.11	31.1	103.0	206.2	1.441	-
50	-58.63	114.98	2699.89	0.4765	0.5500	1.0194	-0.0071	34.6970	2.24	32.5	128.4	218.8	0.563	-
50	-58.63	114.98	3902.44	0.4780	0.5569	1.0290	-0.0059	34.6772	2.25	32.4	138.3	233.3	0.097	-
50	-58.63	114.98	4608.05	0.4719	0.5685	1.0582	0.0179	34.6607	2.25	32.6	138.8	245.7	-0.101	-
53	-60.40	114.99	10.63	0.2715	0.4146	0.7550	0.0689	33.8769	1.69	26.3	26.6	346.2	2.060	-
53	-60.40	114.99	49.95	0.3287	0.4411	0.8619	0.0921	33.9067	1.73	26.7	25.6	350.8	1.497	-
53	-60.40	114.99	90.57	0.3019	0.4375	0.7862	0.0468	33.9767	1.97	28.0	33.9	353.8	0.075	-
53	-60.40	114.99	135.80	0.3299	0.4447	0.8296	0.0550	34.0310	2.05	29.7	40.3	337.9	-0.291	-
53	-60.40	114.99	182.88	0.3500	0.4620	0.8214	0.0094	34.2898	2.30	33.6	59.3	242.6	1.255	-
53	-60.40	114.99	250.94	0.3748	0.4814	0.8675	0.0113	34.4799	2.41	34.8	73.2	193.3	1.929	-
53	-60.40	114.99	300.68	0.3789	0.4798	0.8929	0.0342	34.5356	2.39	34.8	76.4	184.6	2.045	-
53	-60.40	114.99	401.59	0.3911	0.4847	0.8738	-0.0019	34.6058	2.35	33.9	80.6	182.3	2.048	-
53	-60.40	114.99	501.19	0.4187	0.4900	0.9176	0.0089	34.6642	2.28	32.8	83.1	183.3	2.062	-
53	-60.40	114.99	651.48	0.4216	0.4866	0.9030	-0.0051	34.7076	2.20	31.9	85.6	189.5	1.992	-
53	-60.40	114.99	802.48	0.4110	0.4892	0.8955	-0.0047	34.7288	2.17	31.4	87.4	194.7	1.924	-
53	-60.40	114.99	1001.59	0.4198	0.4908	0.9266	0.0160	34.7390	2.15	-	91.1	200.0	1.776	-
53	-60.40	114.99	1502.29	0.4510	0.4893	0.9529	0.0126	34.7329	2.17	31.1	102.5	208.2	1.326	-
53	-60.40	114.99	1801.28	0.4450	0.4871	0.9352	0.0031	34.7231	2.21	31.4	110.7	210.1	1.082	-
53	-60.40	114.99	2402.05	0.4598	0.5067	0.9570	-0.0096	34.7001	2.27	32.4	123.7	218.1	0.627	-
53	-60.40	114.99	3200.52	0.4634	0.5101	0.9726	-0.0009	34.6780	2.30	32.6	131.7	-	0.221	-
53	-60.40	114.99	3900.23	0.4619	0.5177	1.0190	0.0394	34.6737	2.28	32.6	133.6	237.1	0.026	-
53	-60.40	114.99	4530.24	0.4609	0.5199	0.9828	0.0019	34.6629	2.28	32.4	138.2	242.7	-0.083	-
57	-62.42	114.43	11.14	0.3149	0.4155	0.8049	0.0745	33.8384	1.83	27.5	41.6	351.0	0.615	-
57	-62.42	114.43	56.30	0.3058	0.4285	0.7952	0.0608	34.0199	1.94	28.5	50.9	337.7	-0.528	-
57	-62.42	114.43	81.47	0.2974	0.4441	0.8123	0.0708	34.1269	2.05	29.9	57.2	312.0	-0.555	-

station	lat	long	depth	Se(IV)	Se(VI)	Total Se	org Se	botsalt	phos	nit	sil	do	temp	Chl a
57	-62.42	114.43	111.60	0.3058	0.4585	0.7918	0.0275	34.3617	2.24	32.9	70.1	247.7	0.245	-
57	-62.42	114.43	171.78	0.3245	0.4726	0.8437	0.0466	34.5166	2.29	33.5	78.5	210.7	1.056	-
57	-62.42	114.43	250.99	0.3503	0.5157	0.9254	0.0594	34.6336	2.28	33.2	84.4	193.9	1.589	-
57	-62.42	114.43	301.43	0.3340	0.5318	0.8717	0.0059	34.6662	2.25	32.8	86.1	191.8	1.669	-
60	-63.66	112.60	10.38	0.3387	0.4312	0.8060	0.0361	34.0072	1.85	28.2	55.1	347.0	0.276	0.30272
60	-63.66	112.60	40.90	0.3295	0.4019	0.7377	0.0064	34.0281	1.87	28.4	58.3	343.3	0.215	0.32716
60	-63.66	112.60	63.89	0.3179	0.4348	0.8445	0.0917	34.3517	2.08	30.9	73.7	282.2	-0.480	0.64966
60	-63.66	112.60	87.69	0.3004	0.4426	0.8052	0.0622	34.5479	2.21	32.6	84.2	229.6	0.434	0.17194
60	-63.66	112.60	131.03	0.3264	0.4502	0.8015	0.0248	34.6717	2.22	32.7	90.2	203.4	1.248	-
60	-63.66	112.60	181.63	0.3887	0.5584	0.9877	0.0405	34.7052	2.21	32.5	92.4	198.0	1.453	-
60	-63.66	112.60	251.00	0.3977	0.5858	1.0479	0.0644	34.7219	2.20	32.1	94.3	198.7	1.478	-
60	-63.66	112.60	302.83	0.4144	0.6107	1.0744	0.0494	34.7266	2.20	32.1	95.5	200.5	1.461	-
60	-63.66	112.60	401.79	0.4588	0.6313	1.1152	0.0251	34.7312	2.17	31.9	98.3	201.8	1.391	-
60	-63.66	112.60	500.89	0.4635	0.6292	1.1064	0.0136	34.7315	2.18	31.8	101.0	203.9	1.309	-
60	-63.66	112.60	801.44	0.4802	0.7022	1.2444	0.0621	34.7156	2.20	32.1	109.0	209.3	1.038	-
60	-63.66	112.60	1100.40	0.4831	0.6751	1.1841	0.0259	34.7058	2.22	32.5	114.9	213.6	0.744	-
60	-63.66	112.60	2300.66	0.4789	0.6689	1.1841	0.0362	34.6752	2.27	33.0	126.7	232.9	0.005	-
60	-63.66	112.60	3335.78	0.4517	0.6940	1.1549	0.0092	34.6554	2.27	32.7	119.3	247.8	-0.299	-
64	-64.75	111.93	8.93	0.3190	0.4168	0.7927	0.0570	33.8926	1.87	27.5	65.9	350.6	-0.091	0.13116
64	-64.75	111.93	29.71	0.3212	0.4347	0.8036	0.0477	33.9078	1.88	27.6	65.9	349.7	-0.160	0.16384
64	-64.75	111.93	55.37	0.3234	0.4369	0.8046	0.0443	34.3231	2.10	30.2	74.1	292.1	-0.869	0.38032
64	-64.75	111.93	69.74	0.3455	0.4577	0.8457	0.0425	34.4660	2.20	31.6	79.5	260.0	-0.270	1.55618
64	-64.75	111.93	89.78	0.3758	0.4611	0.8502	0.0133	34.5675	2.24	32.2	85.2	234.8	0.233	0.14198
64	-64.75	111.93	119.77	0.3008	0.4932	0.8948	0.1008	34.6313	2.26	32.3	89.0	218.0	0.763	0.07433
64	-64.75	111.93	200.39	0.3712	0.5791	1.0036	0.0532	34.6929	2.22	32.2	93.7	205.3	1.191	-
64	-64.75	111.93	250.06	0.4124	0.6107	1.0735	0.0504	34.7096	2.22	32.0	96.0	204.1	1.266	-
64	-64.75	111.93	302.08	0.4833	0.7420	1.2188	-0.0065	34.7143	2.23	32.0	97.4	204.8	1.243	-
64	-64.75	111.93	400.85	0.4728	0.6842	1.1637	0.0067	-	2.21	31.8	100.4	208.8	1.150	-
64	-64.75	111.93	500.75	0.3711	0.7037	1.1283	0.0535	34.7139	2.22	31.9	103.1	210.6	1.049	-

station	lat	long	depth	Se(IV)	Se(VI)	Total Se	org Se	botsalt	phos	nit	sil	do	temp	Chl a
64	-64.75	111.93	700.64	0.4347	0.7284	1.1472	-0.0159	34.7027	2.25	32.0	109.3	215.4	0.782	-
64	-64.75	111.93	1200.98	0.4480	0.6611	1.1130	0.0039	34.6901	2.27	32.6	121.4	221.0	0.407	-
64	-64.75	111.93	1800.44	0.4381	0.6577	1.1083	0.0125	34.6740	2.29	32.6	122.0	232.4	0.044	-
64	-64.75	111.93	2277.37	0.4598	0.6328	1.0875	-0.0051	34.6621	2.28	32.6	116.9	242.6	-0.232	-
67	-65.22	112.46	10.83	0.2993	0.4345	0.7714	0.0377	33.9098	1.89	28.4	65.1	360.2	-0.357	-
67	-65.22	112.46	30.82	0.3527	0.4340	0.7962	0.0096	33.9694	1.91	28.2	64.2	359.5	-0.540	-
67	-65.22	112.46	50.80	0.3527	0.4950	0.8976	0.0500	34.0569	1.91	28.3	63.5	358.3	-0.655	-
67	-65.22	112.46	100.87	0.3428	0.5160	0.8704	0.0115	34.2279	2.08	30.5	66.8	328.9	-1.703	-
67	-65.22	112.46	150.77	0.3785	0.4647	0.8948	0.0516	34.2869	2.13	31.3	67.2	320.7	-1.795	-
67	-65.22	112.46	200.96	0.4089	0.4953	0.9280	0.0239	34.4187	2.19	31.8	74.0	282.1	-0.934	-
67	-65.22	112.46	251.06	0.4164	0.5314	0.9485	0.0008	34.5295	2.23	32.3	81.3	251.6	-0.035	-
67	-65.22	112.46	300.74	0.3980	0.5927	0.9944	0.0036	34.6076	2.26	32.4	86.6	229.8	0.527	-
67	-65.22	112.46	350.63	0.5028	0.5109	1.0047	-0.0090	34.6619	2.25	32.4	91.1	218.4	0.901	-
67	-65.22	112.46	400.76	0.4873	0.5292	1.0161	-0.0004	34.6652	2.24	32.1	92.6	221.0	0.826	-
67	-65.22	112.46	500.83	0.5315	0.5242	1.0237	-0.0321	34.6699	2.25	32.0	97.1	225.5	0.649	-
67	-65.22	112.46	1001.47	0.4874	0.5772	1.1001	0.0355	34.6729	2.29	32.6	114.8	228.0	0.248	-
67	-65.22	112.46	1327.03	0.4990	0.5967	1.0514	-0.0443	34.6654	2.30	32.7	118.6	237.3	-0.123	-

Appendix 2

Preparation of modified AQUIL medium

Preparation of modified AQUIL medium

The regular AQUIL medium was enriched in Co, Zn, Cu and Ni, while Mo and Se concentrations were decreased to fit concentrations in between AQUIL and artificial seawater recipes. The concentration of HCO_3^- and H_3BO_3 was increased 3–fold to buffer the pH of the medium at 7.9 (with the addition of acidic trace metal solutions). The vitamin stock was modified to the vitamin stock present in f/2 media. Trace metal and EDTA concentrations were homogeneously increased as suggest by Price and Morel (1988/89) minimizing the effect of possible background contamination on the chemical speciation.

Modified AQUIL composition:

Inorganic seawater	[M]	uma	g for 5 L	g for 5L conc. 5x
Solution 1: Salt anhydrous				
NaCl	4.20E-01	5.84E+01	1.23E+02	6.14E+02
Na_2SO4	2.88E-02	1.42E+02	2.05E+01	1.02E+02
KCl	9.39E-03	7.46E+01	3.50E+00	1.75E+01
$NaHCO_3$	7.14E-03	8.40E+01	3.00E+00	1.50E+01
KBr	8.40E-04	1.19E+02	5.00E-01	2.50E+00
H_3BO_3	1.46E-03	6.18E+01	4.50E-01	2.25E+00
NaF	7.14E-05	4.20E+01	1.50E-02	7.50E-02
Solution 2: Salt hydrated				
$MgCl_2.6H_2O$	5.46E-02	2.03E+02	5.55E+01	2.78E+02
$CaCl_2.2H_2O$	1.05E-02	1.47E+02	7.72E+00	3.86E+01
$SrCl_2\ 6H_2O$	6.38E-05	2.67E+02	8.51E-02	4.25E-01

Enrichment		uma	Conc in stock 1000	g/100 ml	
Solution 3: Macronutrients					
$NaNO_3$	3.00E-04	**8.50E+01**	3.00E-01	2.55E+00	
Solution 4: Macronutrients					
$NaH_2PO_4.2H_2O$	1.00E-05	**1.56E+02**	1.00E-02	1.56E-01	
Solution 5: Macronutrients					
$Na_2SiO_3.5H_2O$	1.00E-04	**2.12E+02**	1.00E-01	2.12E+00	
Solution 6: Fe-EDTA (trace metals)		mol/L		ml/100 ml	
$FeCl_3$	2.00E-06	1.79E-02	2.00E-03	1.12E+01	ml
Na_2-EDTA	3.55E-05	2.92E+02	3.55E-02	1 04E+00	g
Solution 7: Trace metals					
$ZnCl_2$	6.00E-07	1.53E-02	6.00E-04	3.92E+00	ml
$CoCl_2$	1.00E-07	1.70E-02	1.00E-04	5.89E-01	ml
$MnCl_2$	1.35E-07	1.82E-02	1.35E-04	7.42E-01	ml
Na_2MoO_4	1.00E-08	1.04E-02	1.00E-05	9.59E-02	ml
$NiCl_2$	6.00E-08	1 70E-02	6.00E-05	3.52E-01	ml
Na_2-EDTA	6.00E-05	2.92E+02	6.00E-02	1.75E+00	g
$CuCl_2$	1.20E-08	1.57E-02	1.20E-05	7.63E-02	ml
Solution 8: Selenium					
Na_2SeO_3	1.00E-09	1.27E-02	1.00E-06	7.90E-03	ml
Solution 9: Vitamin					mg/100ml
Thiamine HCl	2.97E-07	**3.37E+02**	2.97E-04	1.00E-02	1.00E+01
Biotin	4.09E-09	**1.36E+03**	4.09E-06	5.54E-04	5.54E-01
B12	1.47E-09	**2.44E+02**	1.47E-06	3.59E-05	3.59E-02

Note: when uma = bold (salt or vitamin from CSIRO culture collection) otherwise from newly bought salt (Sigma, all puriss p.a., ACS reagent, or ICP metal standard).

Medium preparation (in the semi-clean-room):

All containers and materials (filters, syringe, pipette tips, columns, weigh boat) had to be washed with acid; either using an acid bath or a direct rinse with 10% HCl. They were rinsed 7-times with Milli-Q and dried under laminar flow hood in the semi-clean room.

Stock solutions and media were prepared in the semi-clean-room. Solutions were stored in the cold room (dark) in double plastic bag (ziplock type) in a plastic box.

5 l of inorganic seawater (IS; solutions 1 and 2) was made. Solution 1 and 2 were prepared separately in 2 l of each solution. They were then mixed and diluted to 5 l. 200 g of clean and activated chelex (see below) was added directly to the IS solution. The solution was stirred in presence of the chelex for 1 week at room temperature. The chelex was then removed by filtration (0.45 µm, nitrocellulose membrane).

Stock solutions of macronutrients (solution 3, 4 and 5) were prepared 1000-fold concentrate (100 ml in LDPE bottle) and chelexed separately through a plastic chromatography column at a flow rate of 1 ml min^{-1} (see below).

Stock solutions of trace metals (solution 6 and 7) were not chelexed, they were filtered (see below). To prepare these solutions, first the EDTA was dissolved (at slightly basic pH ca. 8), then the pH was dropped down to 6-7 (Seastar Baseline concentrated HNO_3) and the metals (from ICP standard) were added and pH was adjusted to ca. 4-5. At this pH, the metals and EDTA remain soluble. Selenium was prepared separately (solution 8) to facilitate variations in the selenium concentration. Stock solutions had to be 1000-fold concentrate.

Vitamin-mix (solution 9) was prepared 1000-fold concentrate, non-chelexed but filtered (see below), and stored in 1.5 ml eppendorf tubes in plastic zip-lock bag in the freezer.

<u>Chelex preparation (in the semi-clean room):</u>

Chelex had to be regenerated and prepared (at room temperature) as follows, prior to being used. For 250 g Na-Chelex-100 or approximately 350 ml used chelex in a 1.5 l glass beaker with a large stirrer bar. The conditioning had to be performed under agitation in a fume hood.

1/ Soak for 3-4 hours in methanol (600 ml or at least 2 bed volume) to remove the IDA not bound to the resin.

2/ Rinse with 3 x 1 l Milli-Q.

3/ Soak in 1 M HCl for 3 hours (1 l or minimum 2 bed volume)

4/ Rinse with 5 x 1 L Milli-Q and check the pH with pH paper. Do additional rinses if required to bring to neutral.

5/ Soak in 2 M NH_4 (1 l or 3 bed volume) for 1 week. Due to change in ionic charge the resin increases volume and becomes gelatinous.

6/ Rinse with 5x 1 l Milli-Q, the smell of NH_3 should not be detectable.

7/ Soak in 0.1 M HCl for 10 minutes.

8/ Rinse with 7 x 1 l Milli-Q and then transfer the wet resin into an acid washed plastic bottle.

9/ Rinse with 7 x 1 l Milli-Q adjusted to pH 7.8-8.0, equal to the modified AQUIL, with NaOH (ultrapure). Keep the resin in pH 8 Milli-Q. Sample the water for NH_4 and conductivity. Conductivity should be less than 5 µS and NH_4 should be low (below 0.1 µM).

Binding characteristics of Chelex 100: binding groups considered = IDA (imminodiacetic acid)

Metal	*log K*
Zn	*6.58*
Co	*6.54*
Cu	*10.1*
Fe	*5.45*
Mg	*2.72*
Ca	*2.09*
Na	*0.36*
H	*9.52*
Pb	*6.87*
Cd	*9.26*
Sr	*2.23*

Ionic strength = 1 M, 25 °C (NIST ver. 8.0)

Chelexing the solutions (in the semi-clean-room):

Before its use, the chelex had to be soaked overnight in the same medium that would be passed through (2 bed volume minimum). The chelex was rinsed once with 1-2 bed volume of the solution that was also discarded. The chelex could then be used to collect trace metal clean solutions.

For the inorganic seawater (IS): ca. 200 g of resin had to be used in 5 l. The resin was directly added to a carboy containing the 5 l of IS and a large stirrer bar. The resin was soaked for 1 week in the IS at room temperature. The resin was then removed by a 0.45 µm filtration (plastic filtration device). The IS was stored in the dark in the cold room in

double plastic bags and a plastic box. Trace metal contamination was then verified by ICP-MS.

For the 100 ml macronutrient stock solutions, trace metal decontamination was performed in a plastic column. The plastic column must have been soaked in 0.1% HCl overnight and rinsed 7 times with Milli-Q prior being used. The resin was loaded into the plastic columns and excess of solution was passed through and discarded. The solution was passed through the column at a flow rate of 1 ml min^{-1}. Typically, 2-3 g of chelex was enough for 100 ml solution (contamination level should be much lower than in the IS, since the amount of salt added are much lower). Trace metal contamination was checked for each chelexed solution (by ICP-MS). Solutions were stored in the fridge in double plastic bags.

Note: The used chelex was stored in a plastic bottle in Milli-Q at room temperature to be regenerated. Allow a maximum of 3-4 regenerations. Prior to use, the ability of the chelex to remove Cu (50 nM passed at a flow rate of 3 and 1 ml min^{-1}) would be verified. The flow rate of 1 and 3 ml min^{-1} decrease the Cu concentration by 370- and 40-fold, respectively.

Sterilisation (in lab culture room and semi clean-room):

Inorganic seawater enriched with macronutrients needs to be microwaved. The microwave used was in the Chemistry lab, close to the laminar flow hood. Sixty millilitre of culture media (IS enriched in macronutrients) is microwaved at low intensity (90 W) for 4 successive heating steps (3 min, 2 min, 20 s, and 10 s). The culture flask is sealed and shaken between each step. The final temperature is 96°C. Microwave heating was adjusted to 3 min, 2 min, 20 s, and 20 s for 75 ml culture media in a polycarbonate erlenmeyer (125 ml). The culture flasks were allowed to cool down at room temperature in the laminar flow hood. Procedure efficiency was verified using mix of non axenic cultures (*Emiliana huxleyi*, *M. pusilla*, *C. muelleri*) in 1:1:1 ratio. Subsequent to microwaving, no algal or bacterial growth could be seen on agar plate (checked for 2 weeks at 25°C). In addition, freshly (24 h) sterilized IS with trace metals and vitamin addition was spread in a seawater agar plate. No bacterial contamination could be seen after 1 week at 25°C.

Note: Add 240 µl of solution of $NaHCO_3$ and H_3BO_3 for 60 ml solution and 300 µl for 75 ml media. This ensures buffered pH of 7.9 even after trace metal additions. The solution of $NaHCO_3/H_3BO_3$ was chelexed as for macronutrients. This solution needed to be added prior to microwaving.

Trace metals and vitamin solutions were 0.2 µm filtered. Care was taken not to touch the solution with the black rubber of the syringe (large Zn contamination). To be done in the semi-clean-room.

Trace metals and vitamins were added after microwave sterilisation of the media. The media was stored 1 week at incubation temperature (in double sealed plastic zip bags) to allow equilibration.

Background verification (monthly):

Contamination was verified on a regular basis by direct application of the medium to an agar plate (seawater agar with F/2 enrichment). Medium composition would be checked by ICP-MS for trace metals and major nutrient accuracy.

The rinsing solution, NaCl 0.6 M and $NaHCO_3$ 2.38 mM (pH ca. 8), was chelexed. The chelexed rinsing solution was also kept for a maximum of 2 months to prevent trace metal contamination due to manipulation.